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STUDY OF THE INTERPLAY BETWEEN TET PROTEINS AND MICRORNAS FOR MEMORY FORMATION

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“On exige de l'explorateur qu'il fournisse des preuves. S'il s'agit par exemple de la découverte d'une grosse montagne, on exige qu'il en rapporte de grosses pierres.”

Antoine de Saint-Exupéry, Le Petit Prince

À mes parents, Simone et Quirin

À mes grands-parents Jeanne, Hélène et Armand

“Es irrt der Mensch, so lang er strebt”

Johann Wolfgang von Goethe – Faust, Prolog im Himmel

Contents

Abbreviations

3'UTR	three prime untranslated transcribed region
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5hmU	5-hydroxymethyluracil
5mC	5-methylcytosine
Aβ	Amyloid β
AD	Alzheimer's disease
AID	Activity induced deaminase
AMPA	α-amino-3-hydroxy-5-methyllisoxazolepropionic acid
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
APP	Amyloid precursor protein
AVP	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
BER	Base excision repair
C	Cytosine
CA1	Cornu ammonis 1
CaMK	Calcium/Calmodulin dependent kinase
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
Cdk5	Cyclin dependent kinase 5
C/EBP	CCAAT-enhancer-binding protein
CFC	Contextual fear conditioning
ChIP	Chromatin immunoprecipitation
CpG	Cytosine-guanine dinucleotide
CGI	CpG island
CNS	Central nervous system
CRE	cAMP-response element
CREB	cAMP-response element binding protein
DMR	Differentially methylated region
DNMT	DNA methyltransferase
DGCR8	DiGeorge syndrome critical region gene 8
ESC	Embryonic stem cell
FGF1	Fibroblast growth factor 1
FTLD	Frontotemporal lobar degeneration
FXTAS	Fragile X tremor/ataxia syndrome
GR	Glucocorticoid receptor
HAT	Histone acetyl transferase

HDAC	Histone deacetylase
HDM	Histone demethylase
HMT	Histone methyltransferase
IEG	Immediate early gene
IIPFC	Infralimbic prefrontal cortex
KO	Knockout
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
L-VSCC	L-type voltage sensitive calcium channel
MBD	Methyl-binding domain
MBP	Methyl-binding protein
MDD	Major depressive disorder
MeCP2	Methyl-CpG-binding protein 2
miRNA	Micro-RNA
mOSN	mature olfactory sensory neurons
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
MWM	Moris water maze
Ncor	Nuclear receptor co-repressor
ncRNA	Non-coding RNA
NF-KB	Nuclear factor-kappa B
NFT	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
piRNA	piwi RNA
PKA	Protein kinase A
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
PS1	Presenilin 1
PTM	Post-translational modification
RBP	RNA-binding protein
REST	RE1-silencing transcription factor
miRISC	miRNA-induced silencing complex
RS	Rett syndrom
SAM	S-adenosylmethionine
SSRI	Selective serotonin re-uptake inhibitor
STM	Short-term memory
SUMO	Small-ubiquitin like modifier
TDG	Thymine DNA glycolase
TDP-43	TAR DNA binding protein of 43 kDa
TET	Ten-eleven translocation methylcytosine dioxygenase
TF	Transcription factor

TRBP	TAR RNA binding protein
TRD	Transcriptional repressor domain
TSS	Transcription start site

Gene/Protein nomenclature

Mouse/Rat

In this thesis, mouse gene symbols are italicised, with only the first letter in uppercase and the remaining letters in lowercase (*Bdnf*). Protein designations are the same as the gene symbol, but are not italicised and all letters in uppercase (BDNF) according to the guidelines for mouse and rat gene nomenclature.

Human

In this thesis, human gene symbols are italicised, with all letters in uppercase (*BDNF*). Italics are not necessary in gene catalogs. Protein designations are the same as the gene symbol, but are not italicised, with all letters in uppercase (BDNF) according to the guidelines for human gene nomenclature.

Abstract

Epigenetic mechanisms are critical regulators of gene expression underlying learning and memory formation. One of the most widely studied epigenetic mechanism is the methylation of DNA, and until recently, was regarded as stable in post-mitotic cells, such as neurons. This view has, however, recently been actualized, as DNA methylation was suggested to be dynamically regulated in a locus-specific manner upon neuronal stimulation and learning. Consistently, the expression of DNA methylases also appears to be dynamically regulated in the brain. A family of DNA demethylases, Ten-eleven translocation (TET) proteins (TET 1, 2 and 3) mediate the conversion of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC). There is accumulating evidence that TET proteins, and hence 5mC and 5hmC levels, are regulated by neuronal activity to control gene transcriptional regulation and memory. However, little is currently known about the mechanisms bringing about their dynamic regulation.

The scope of the present work was to explore the modes of regulation of TETs in the context of neuronal activity and memory formation. Specifically, the implication of microRNAs (miRNAs), a class of short non-coding RNAs capable of modulating gene expression rapidly and reversibly, in controlling TET expression is investigated. The results indicate that *Tet3* is preferentially up regulated in response to learning *in vivo*. Consistently, neuronal activation triggers increased *Tet3* gene expression *in vitro*. Furthermore, targeted transcriptional analysis revealed that memory-related genes, such as *Creb1*, are sensitive to changes in TET3 levels. This suggests that activity-dependent TET3 regulation in the hippocampus can affect the transcriptional activity of genes related to learning and memory formation.

miR-29b, a miRNA whose sequence is complementary to multiple sites in *Tet*s 3' untranslated transcribed regions (3'UTRs), is inversely regulated in

response to learning and neuronal activation. Importantly, miR-29b binds to the 3'UTRs of all Tets and controls their expression, though, a preference towards *Tet3* is observed at low concentration. Overall, these findings suggest that miRNAs play a role in the activity-dependent regulation of TETs associated with learning and memory formation.

Next, SAM68, a nuclear RNA-binding protein previously known to regulate alternative splicing, was identified as an important regulator of miR-29b biogenesis at the transcriptional level. In addition, *Sam68* was found to be a target of TET3, which suggests that the expression of *Sam68*, miR-29b, and TET3 might be intricately regulated in response to neuronal activity through a feed-back loop.

In summary, this study identifies a novel molecular pathway involving the miR-29 cluster and the RNA-binding protein SAM68 in the regulation of the DNA demethylase TET3. This regulatory mechanism may contribute to the epigenetic control of genes underlying memory formation.

Résumé

Les mécanismes épigénétiques sont des régulateurs essentiels de l'expression des gènes dans le cas de l'apprentissage et de la formation de la mémoire. L'un des mécanismes épigénétiques le plus fréquemment étudié est la méthylation de l'ADN, qui jusqu'à présent, était considérée comme stable dans les cellules post-mitotiques telles que les neurones. Cependant, cette opinion a récemment été reconsidérée car la méthylation de l'ADN semblerait être régulée de façon dynamique au niveau de certains gènes suite à l'activité neuronale et l'apprentissage. L'expression des ADN méthyltransférases est également régulée de façon dynamique dans le cerveau. Les protéines TET (TET1, 2 and 3) (ten-eleven translocation) sont une famille d'ADN déméthylases capables d'hydroxyler une cytosine méthylée (5mC) en 5-hydrométhylcytosine (5hmC). Des données de plus en plus nombreuses suggèrent que les protéines TETs, ainsi que les niveaux de 5mC et 5hmC, sont régulés par l'activité neuronale afin de contrôler la régulation transcriptionnelle et la mémoire. En revanche, les mécanismes sous-jacents à leur régulation sont à ce jour méconnus.

Le sujet de cette dissertation est d'explorer les modes de régulation des protéines TET dans le contexte de l'activité neuronale et de la formation de la mémoire. Précisément, il s'agit d'étudier l'implication des microARNs, une classe de petits ARNs non codants capable de rapidement et réversiblement moduler l'expression des gènes, dans le contrôle de TETs. Les résultats indiquent que le gène *Tet3* est préférentiellement surrégulé in vivo suite à l'apprentissage. Aussi, l'activité neuronale induit une augmentation de l'expression du gène *Tet3* in vitro. De plus, une analyse transcriptionnelle ciblée a démontré que les gènes associés à la mémoire, comme *Creb1*, sont sensibles au niveau d'expression de *Tet3*. Ceci suggère que la régulation de *Tet3* dans l'hippocampe en fonction de l'activité, peut conduire à la modification de l'activité transcriptionnelle des gènes liés à l'apprentissage et à la formation de la mémoire.

miR-29b, un microARN dont la séquence est complémentaire à plusieurs sites dans la région 3' non traduites (3'UTR) de *Tet3*, est inversement régulé en réponse à l'apprentissage et l'activité neuronale. Par ailleurs, miR-29b se lie à la région 3'UTR des *Tets* et contrôle leur expression, mais présente une préférence pour *Tet3* à faible concentration. Globalement, ces observations suggèrent que les microARNs jouent un rôle dans la régulation de TETs associé à l'apprentissage et la formation de la mémoire.

Par la suite, SAM68, une protéine nucléaire se liant à l'ARN généralement impliquée dans le contrôle de l'épissage alternatif, a été identifiée comme un important régulateur transcriptionnel dans le contrôle de la biogénèse du miR-29b. Ceci suggère que l'expression des gènes codant pour SAM68, miR-29b and TET3 est régulée en réponse à l'activité neuronale par le biais d'une boucle de rétroaction.

En résumé, cette étude a permis l'identification d'une nouvelle voie moléculaire impliquant le microARN-29 et SAM68 dans la régulation de l'ADN déméthylase TET3. Ce mécanisme de régulation contribue probablement à la régulation transcriptionnelle des gènes nécéssaires à la formation de la mémoire.

1 Introduction

1.1 Understanding memory

"In order to be yourself, you have to remember who you are."

Joseph LeDoux

The ability to learn, retain and recall information over time is a key feature of cognitive functions in mammals. In fact, learning offers us the possibility of acquiring new knowledge, and memory gives us the capability to remember past experiences and recall to mind previously learned facts, impressions, skills and habits.

Based on its temporal persistence, memory can be classified in to short-term, lasting minutes to hours, or long-term, lasting days, months and even years. Long-term memories further include declarative memories, memories of facts and events, places and objects; and non-declarative memories, memories involved in perceptual and motor skills. These two types of memory also differ with regard to the brain regions involved. Whereas declarative memory mostly depends on the hippocampus and related cortical areas, non-declarative memory implicates other brain regions, namely the cerebellum, the striatum and the amygdala.

Memory comprises of many processes: acquisition, which is the encoding of new information; consolidation, which is the process by which short-term memory (STM) is stabilized into a persistent long-term memory (LTM) and retrieval, which refers to the recall of the memory trace (Abel and Lattal, 2001). In addition, memory can further be changed during reconsolidation, as newly consolidated memory may be subjected to modifications through reminders and interference when it is retrieved (McKenzie and Eichenbaum, 2011).

Classical fear conditioning in rodents is a valuable paradigm for exploring memory processing at the molecular level. In contextual fear conditioning (CFC), a mouse is placed in a conditioning chamber (context) where it receives a foot shock and learns to associate the context with the shock (memory acquisition). The mouse is then returned to its homecage, where the stabilization of the fear association into long-term memory is consolidated (memory consolidation). Retrieval occurs when the animal is placed back in the context in the absence of shock. Re-presentation of the context elicits freezing, which is a characteristic fear response in rodents (memory retrieval). Retrieval tests do not only reactivate the memory from acquisition (memory re-consolidation), but also lead to the establishment of new memories. The original learning can be gradually suppressed as the animal learns that the context no longer predicts the shock (memory extinction) (reviewed in Abel and Lattal, 2001).

The use of pharmacological and lesion approaches in fear conditioning experiments has greatly contributed to our understanding of the brain regions and temporally distinct memory processes involved in associative learning. Multiple studies have demonstrated that the hippocampus is a critical structure underlying contextual fear memory. Electrolytic lesions of the dorsal hippocampus (Maren et al., 1997; Phillips and Ledoux, 1992) or pharmacological manipulation prior to training produce deficits in both acquisition and retrieval of contextual fear memory (Matus-amat et al., 2004; Sanders and Fanselow, 2003). Furthermore, post training hippocampal lesions tremendously affect contextual fear memory (Kim and Fanselow, 1992). This means the hippocampus stores the information only for a limited time after conditioning. Memory consolidation involves the transfer of information to other brain regions such as the neocortex (Anagnostaras et al., 1999).

1.2 Molecular basis of memory

The identification and characterization of the molecular mechanisms underlying memory formation in the hippocampus have strongly benefited from the study of cellular models of learning, including certain forms of synaptic plasticity. At the cellular level, the formation of memory is accompanied by activity-dependent changes in synaptic transmission, a phenomenon that is referred to as synaptic plasticity (Neves et al., 2012). Various types of plasticity can be modeled in the mammalian hippocampus by applying electrical or chemical stimulation, and subsequent recording of synaptic transmission. Following neuronal stimulation, synaptic transmission can either be enhanced in the case of long-term potentiation (LTP) or repressed in the case of long-term depression (LTD) (Ho et al., 2011).

There is evidence that plasticity is important for immediate learning. Notably, STM is mediated by transient modifications of preexisting synaptic proteins, i.e. (de)phosphorylation of enzymes, receptors and ion channels. These modifications ultimately alter the efficiency of synaptic transmission. In contrast, the consolidation of this plasticity into LTM is generally thought to depend on the activation of second messengers that initiate both gene transcription and the synthesis of new proteins (Kandel et al., 2014).

Typically, release of the excitatory neurotransmitter glutamate from the presynaptic neuron and its binding to N-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on the postsynaptic neuron cause the opening of these ionotropic glutamate channels. Then, membrane depolarization occurs, causing the opening of L-type voltage sensitive calcium channels (L-VSCCs) and increased calcium concentrations in the postsynaptic neuron (Greer and Greenberg, 2008). Calcium influx through NMDA receptors and L-VSCCs is critical for activity-dependent gene transcription, and promotes long-term plasticity. Depending on the route and dynamics of calcium entry (either through NMDA receptors

or L-VSCCs), a specific gene expression response is triggered in the nucleus via the activation of a range of signaling molecules such as $Ca^{2+/}$Calmodulin dependent kinase II (CamKII), protein kinase A (PKA), protein kinase C, and calcineurin (Kandel, 2012). These signaling molecules are mainly composed of kinases and phosphatases that activate directly or indirectly various synaptic proteins and transcription factors (TFs). For example, CamKII promotes phosphorylation of AMPA receptors, thereby increasing channel conductance, thus enhancement of synaptic transmission, as well as accumulation of AMPA receptors into the synaptic membrane (Shepherd and Huganir, 2007).

A well-characterized TF implicated in synaptic plasticity and memory in a variety of systems is the cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB) (reviewed in (Carlezon et al., 2005)). For instance, CREB phosphorylation promotes gene transcription of cAMP response element (CRE)-dependent genes in combination with several cofactors (Lonze and Ginty, 2002). Notably, the nuclear activation of TFs leads to the expression of a multitude of genes. Some of them are rapidly and transiently induced such as the immediate early genes (IEGs). Many IEGs, including c-Fos and Zif/268 act as TFs themselves, thus alter neuronal function based on their downstream targets. As a result, a tightly coordinated gene expression program is induced that promotes dendritic and axonal growth, synapse development, and neuronal plasticity. In addition, many activity-dependent genes encode effector proteins that directly control various aspects of neuronal function, such as the brain derived neurotrophic factor (*BDNF)*. Following transcription, the newly transcribed messenger RNAs (mRNAs) are transported to the translational machinery, in the neuronal soma and synapses for subesequent protein synthesis to occur. Newly synthesized proteins contribute to the restructuration of existing synapses and generation of new neuronal circuits (Sutton and Schuman, 2006).

Studies examining the molecular basis of memory have found many of the

signaling molecules involved in synaptic plasticity to be important for memory acquisition and consolidation. Inducible deletion of NMDA receptor function in hippocampal area cornu ammonis 1 (CA1) disrupts the consolidation of contextual memories, but not the retrieval of these memories (Shimizu et al., 2000). Administration of protein synthesis inhibitors after training affects LTM for contextual fear conditioning, indicating that the synthesis of new proteins is critical for contextual memories (Abel et al., 1997). For protein synthesis to occur, long-term contextual memory storage requires the activation of the cAMP/PKA/CREB pathway. In fact, a reduction in PKA hippocampal activity in forebrain neurons leads to selective deficits in LTM (Abel et al., 1997). During consolidation of contextual fear memories, two time periods of sensitivity to inhibitors of PKA and protein synthesis have been described: one shortly after conditioning, and another four hours later (Bourtchouladze et al., 1998). This indicates that there are waves of gene expression and protein synthesis that occur in the brain during memory acquisition and consolidation. CREB is activated after training (Bernabeu et al., 1997), and disruption of CREB alpha and gamma isoforms leads to impairments in the consolidation of fear memories (Bourtchouladze et al., 1994). Similarly, the induction of IEGs such as *c-Fos*, *Arc* and *Bdnf* observed in the hippocampus after CFC potentially underlies protein synthesis-dependent memory consolidation (Hall et al., 2000; Huff et al., 2006). Importantly, gene expression and subsequent protein synthesis are tightly controlled processes that are regulated at multiple levels including transcription, translation as well as mRNA and protein transport and stability.

1.3 Mechanisms of regulation of gene expression

Gene transcription is a sophisticated multi-step cellular process mediated by the collective action of a complex enzymatic machinery comprising the RNA polymerase II along with sequence-specific DNA binding TFs and a number of co-regulators (reviewed in (Kadonaga, 2004)). TFs

function as coordinators between the transcriptional machinery and the information encoded in the DNA sequence. The recruitment of co-regulators (coactivators and corepressors) by TFs to proximal promoter and distal regulatory regions (enhancers and silencers) regulates directly and indirectly the activity of the basal transcriptional machinery. Some of these co-regulators involve chromatin-remodeling complexes and histone-modifying enzymes, which mediate local chromatin alterations to either facilitate or inhibit transcription. Mechanisms that can affect the functional state of regulatory regions via changes in the chromatin architecture without altering the DNA sequence itself are referred to as epigenetic mechanisms of gene regulation.

1.3.1 Epigenetic mechanisms - histone modifications

In eukaryotes, chromatin is organized into nucleosomes that consist of DNA wrapped around octamers of histone proteins (H2A, H2B, H3, and H4 linked by H1). Its structure is highly dynamic and is in part, regulated by histone-modifying enzymes, which covalently modify the amino-terminal tails of histone proteins. Notably, histones can be subjected to a number of posttranslational histone modifications (PTMs), including acetylation, phosphorylation, methylation, ubiquitination and sumoylation of different amino-acids. These modifications are brought about by histone acetyltransferases/deacetylases (HATs/HDACs), histone methyltransferases/demethylases (HMTs/HDMs), and protein kinases/phosphatases, respectively. Depending on the composition of modifications on a given histone, the chromatin architecture, and thus, the accessibility of DNA to the transcriptional machinery may be affected (Kouzarides, 2007).

1.3.2 Epigenetic mechanisms - DNA methylation

Another layer of epigenetic regulation includes the methylation of DNA. DNA methylation is a covalent DNA modification resulting from the addition of a methyl residue at the 5' position of the cytosine pyrimidine ring. Together with DNA methylation, histone PTMs establish a dynamic epigenetic code specific for each gene and for each cellular state, and that determines whether the chromatin is in an open or condensed configuration for gene transcription or silencing (Kouzarides, 2007).

5-methylcytosine (5mC) occurs primarily within the context of CpG dinucleotides in the mammalian genome, although CpH methylation (H is A, C, or T) has recently been identified (Guo et al., 2014; Lister et al., 2013). Globally, CpG dinucleotides are under-represented in the mammalian genome, but 60-90% of all CpGs are methylated in mammals (Deaton and Bird, 2011). However, certain genomic regions known as CpG islands (CGIs) contain a high density of CpG dinucleotides and often overlap with transcription start sites (TSSs) (Bird, 1986). CGI methylation in proximity of the TSS is associated with robust transcriptional silencing. Gene repression seems to be mediated by recruitment of repressive methyl-CpG binding domain (MBD) proteins, such as the methyl-CpG-binding protein 2 (MeCP2) (Klose and Bird, 2006). MBD proteins are thought to interact with HDACs and to establish a repressed chromatin environment. Additionally, the generation of 5mC within gene regulatory elements may prevent the binding of methyl-sensitive TFs, thereby blocking transcription initiation (Liu Y, 2013). In contrast, methylation in the gene body positively correlates with expression (Bender et al., 1999). Interestingly, previous work suggests that gene body methylation results in suppressing intragenic promoters in mammalian cells (Maunakea et al., 2010) and regulating alternative splicing (Maunakea et al., 2013), albeit the role of methylated intragenic CGIs is still unclear. Overall, methylation is key to multiple biological processes, including chromosome X

inactivation, imprinting, suppression of transposons and repetitive elements (Jaenisch and Bird, 2003).

Methylation of cytosines is catalyzed by DNA methyltransferases (DNMTs), including DNMT1, DNMT3a and DNMT3b. *De novo* methyltransferases DNMT3a and DNMT3b are responsible for the establishment of novel DNA methylation patterns (Okano et al., 1999). Conversely, DNMT1 shows high affinity for hemimethylated CpGs, thereby acting as a maintenance methyltransferase that preserved methylation patterns throughout cell division (Bestor, 2000). The idea that DNA methylation patterns are conserved has been recently challenged because of the discovery of the ten-eleven translocation (TET) family of proteins (TET 1, 2 and 3) (Tahiliani et al., 2009). Although it is known that 5mC can be passively lost upon cell division, the identification of TET proteins provides a potential mechanism by which active DNA demethylation may occur. Specifically, TETs have the potential to oxidize 5mC, yielding 5-hydroxymethylcytosine (5hmC), a modified base that has first been identified in the mammalian genome about 40 years ago (Penn et al., 1972).

Although not fully understood, the mechanisms of active DNA demethylation have been proposed to depend on a succession of biochemical reactions catalyzed by several specific enzymes (reviewed in (Wu and Zhang, 2014). 5mC can be oxidized into 5hmC by TETs then deaminated by activation-induced deaminase/apolipoprotein B mRNA/editing enzyme complex (AID/APOBEC) deaminases generating 5-hydroxymethyluracil (5hmU). 5hmU can be then excised by glycosylases and repaired by components of the base excision repair (BER) pathway (Guo et al., 2011a). As AID/APOBEC preferentially deaminates unmodified cytosines due to low activity towards 5mC, and no detectable activity towards 5hmC (Nabel et al., 2012), an alternative deamination-independent pathway has been proposed. It involves TETs-mediated iterative oxidation of 5hmC into 5-formylcytosine (5fC) and 5-

carboxylcytosine (5caC), followed by subsequent excision of 5caC by thymine DNA glycolase (TDG) (He et al., 2011; Ito et al., 2011) (Figure 1.1).

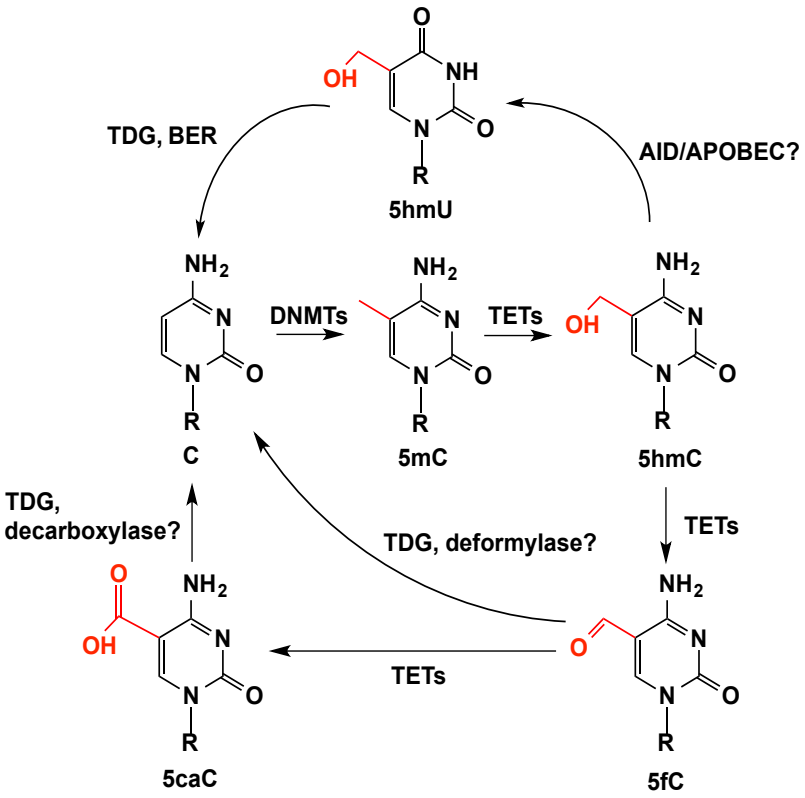

Figure 1-1 Model pathway for active DNA demethylation. DNA methyltransferases (DNMT1, 3a/b) catalyze the methylation of cytosines and lead to the formation of 5-methylcytosine (5mC). 5hmC is generated by oxidation of 5mC by TET proteins (TET1, 2 and/or 3). TETs oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which are then excised by a putative decarboxylase/deformylase or thymine-DNA glycosylase (TDG) through base excision repair (BER) mechanisms. Alternatively, 5-hmC may be deaminated into 5-hmU by AID/APOBEC deaminases, and removed by TDG through BER mechanisms. Relevant modifications are highlighted in red.

1.3.3 DNA hydroxymethylation – a novel epigenetic mark?

Initially thought to be a mere by-product of oxidative stress, 5hmC is now recognized to be a functional DNA mark that plays an important role in various biological processes. Although global 5mC levels are more or less similar across various tissue types, 5hmC content is variable. Importantly, the highest

prevalence of 5hmC has been found in the brain (Globisch et al., 2010; Kriaucionis and Heintz, 2009; Münzel et al., 2010). In addition, global 5hmC levels in human tissue often do not correlate with global 5mC levels (Nestor et al., 2011), suggesting that 5hmC has its own mechanisms of regulation. The relatively high levels of 5hmC in embryonic stem cells (ESCs) during early development (Ito et al., 2010) and in some differentiated cells, in particular neurons (Hahn et al., 2013), suggest that 5hmC represents a stable DNA modification.

The functions of 5hmC are not fully understood but there is accumulating evidence that it might be important for transcriptional regulation. Similar to 5mC, 5hmC is present in CpGs in both the human and in the mouse genome (Lister et al., 2013). Genome-wide mapping in mouse and human ESCs has revealed that 5hmC marks important genomic regions, such as gene bodies, promoters and enhancers (Ficz et al., 2011; Pastor et al., 2011; Stroud et al., 2011; Williams et al., 2011; Wu et al., 2011; Xu et al., 2011). In the human frontal cortex, 5hmC is largely absent within intergenic regions, enriched within gene bodies, and, compared to 5mC, it is more frequently targeted to promoters (Jin et al., 2011). Whole genome mapping at single base resolution confirmed that 5hmC is localized within enhancers and the gene body in fetal and adult mouse brain (Lister et al., 2013). In addition, intragenic 5hmC appears to be enriched at exons compared to introns (Szulwach et al., 2011). Numerous studies have reported a robust positive correlation between intragenic 5hmC levels and gene expression, as well as chromatin accessibility (Colquitt et al., 2013; Jin et al., 2011; Lister et al., 2013; Mellen et al., 2012). Also, the presence of 5hmC within TSS is associated with gene expression levels in the case of promoters with low CpG content, but not for promoters with intermediate or high CpG content (Jin et al., 2011). There is also a significant difference in 5hmC and 5mC abundance between the sense and antisense strand, with 5mC enrichment on the antisense strand and 5hmC enrichment on the sense strand (Wen et al., 2014).

Regulation of gene activity by 5hmC has also been suggested to involve methyl-CpG binding proteins (MBP). MBD3, a member of the MBD family with low binding affinity towards 5mC, can bind to sequences enriched in 5hmC in ESCs (Yildirim et al., 2011). The protein MeCP2 (another MBP) can also associate with 5hmC, although it binds to 5mC with much higher affinity (Mellen et al., 2012; Spruijt et al., 2013). Consistently, MBD1, MBD2, MBD4, and MeCP2 localizations also correlate with the presence of 5hmC in ESCs (Baubec et al., 2013). Several other 5hmC reader proteins have recently been identified in mouse ESCs and neurons by mass spectrometry-based proteomics (Spruijt et al., 2013). Oxidation of methyl residues could also play a role in preventing 5mC-mediated silencing. In fact, the presence of 5hmC strongly inhibits the binding of MBD1 and MBD2, two MBPs that recruit HDACs and HMTs to 5mC (Hashimoto et al., 2012). Overall, these findings suggest that 5hmC may be involved in global epigenetic regulation.

Furthermore, profiling of the TET1 genomic distribution has revealed that TET1 is preferentially bound to the promoters of both repressed and actively transcribed genes in ESCs (Ficz et al., 2011; Pastor et al., 2011; Williams et al., 2011; Xu et al., 2011). As 5hmC is enriched within these regions, these findings corroborate the idea that TET proteins regulate 5hmC generation. Specifically, 5hmC and TET1 are co-enriched at promoters that carry the transcription repressive mark H3K27me3 or the permissive mark H3K4me3 (bivalent promoters) (Pastor et al., 2011; Wu et al., 2011; Xu et al., 2011). Since 5mC is rare in such promoters, it is likely that 5hmC and TET1 are responsible for the regulation of these promoters, in particular in pluripotent and developmentally regulated genes in ESCs. More recently, accumulation of 5hmC and TET3 was associated with a reduction in the heterochromatin mark H3K9me3 and increase occupancy of the TF SP1 within the mouse prefrontal cortex (Li et al., 2014b). Given that both 5mC and 5hmC are abundant in the central nervous system (CNS) and associated with different aspects of transcriptional regulation, much effort has been made to understand the role of these DNA marks in neuronal function.

1.4 DNA modifications in the brain and memory

1.4.1 DNA methylation and DNMTs in the brain

Methylated DNA has traditionally been viewed as a stable epigenetic mark. However, in many cells, including post-mitotic cells like adult neurons, DNA methylation and demethylation occur in a dynamic manner. The first experimental evidence linking DNA methylation to brain activity was provided by Vanyushin and co-workers in 1977 who showed that active avoidance and food-seeking behaviors in rats affect the global level of 5mC within regions of the brain critical for memory formation, such as the hippocampus and the neocortex (Guskova et al., 1977). Many years later, the topic regained momentum upon the discovery that neuronal stimulation induces methylation/demethylation of many CpGs in different loci, such as the promoter of *Bdnf* and fibroblast growth factor-1 (*Fgf1*) (Ma et al., 2009). In this case, demethylation correlates with increased mRNA and protein expression, possibly due to the dissociation of repression complexes, *i.e.*, MeCP2-Sin3 at the *Bdnf* promoter (Martinowich et al., 2003). Furthermore, DNA methylation mapping using methylation sensitive restriction enzymes have revealed that many genes undergo active DNA methylation and demethylation *in vivo* after neuronal activation in the adult mouse brain (Guo et al., 2011b). In this experiment, the methylation status of at least 1.4% CpGs was found to be changed. The methylation changes in the vicinity of putative promoters were anticorrelated with changes in gene expression. Consequently, DNA methylation in conjunction with chromatin remodeling appear to be key processes in the regulation of gene transcription in response to neuronal activity.

DNA methylation and demethylation are also dynamically modulated during learning and memory formation in the adult rodent brain. Following CFC, DNA methylation increases within the promoter of the memory suppressor gene

PP1, but decreases at the promoter of *Reelin*, a memory-activating gene. In the hippocampus, these changes occur within 30 minutes after training but they are transient, as DNA methylation reaches baseline level after 24 h (Miller and Sweatt, 2007) . The same group has reported similar findings in the context of *Bdnf* regulation. Notably, *Bdnf* mRNA gradually increased in the area CA1 of the hippocampus in response to CFC and returned to baseline after 24h. CFC triggers methylation changes at multiple CGIs within the *Bdnf* locus (Lubin et al., 2008). In response to activity, methylation changes in the hippocampus appear to be rapid and transient. In contrast, changes in DNA methylation in the cortex persist and remain for several days. Shortly after training, the methylation profile of the gene coding for the phosphatase and memory suppressor calcineurin remains unaltered in the rodent prefrontal cortex. Within 1 day of training, however, the gene encoding calcineurin is subjected to a significant increase of 5mC content in its promoter region, and this effect is still detectable 30 days after training (Miller et al., 2010). More recently, a cell-specific genome-wide profiling of DNA methylation has pinpointed differentially methylated regions (DMRs) in neurons during memory consolidation and maintenance. These DMRs are preferentially located in intergenic and intronic regions, in particular in functional *cis*-regulatory regions. Neuron-specific genes are more likely to contain DMRs than non-specific genes, and DMRs are enriched in genes involved in the CREB and PKA signaling cascades (Halder et al., 2016). These results suggest that both DNA methylation and demethylation are likely to contribute to gene regulation during memory formation.

The mechanisms of DNA methylation/demethylation during memory formation are, at present, not fully understood. There is, however, accumulating evidence that DNMTs are involved in these processes. For example, the mRNA of *de novo Dnmts*, *Dnmt3a/b*, increases in the mouse hippocampus after CFC (Miller and Sweatt, 2007). Similarly, cortical *Dnmt1* is subjected to activation in response to environmental enrichment (Rampon et al., 2000). Furthermore, pharmacological activation of PKC in hippocampal slices leads

to the upregulation of *Dnmt3a* mRNA specifically in the area CA1, suggesting that the PKC signaling cascade activates *Dnmt3a* expression (Levenson et al., 2006). In CFC, interfering with hippocampal DNMT activity using pharmacological inhibitors impairs LTM when delivered before or immediately after training (Lubin et al., 2008; Miller and Sweatt, 2007). Infusion of DNMT inhibitors prevents active methylation of genes known to be involved in memory formation, resulting in aberrant transcription levels (Miller and Sweatt, 2007). Likewise, inducing a deficiency in DNMT1 and DNMT3a by conditional knockout (KO) in forebrain neurons impairs LTM in adult mice (Feng et al., 2010). Furthermore, DNA methylation is important for synaptic plasticity, a cellular process that results in changes in synaptic strength and is thought to contribute to learning and memory. Induction of LTP in rodent hippocampus is blocked in the presence of DNMT inhibitors (Levenson et al., 2006). Likewise, hippocampal LTP is affected in DNMT1 and DNMT3a double KO mice (Feng et al., 2010). These findings indicate a link between DNA methylation, DNMTs, synaptic plasticity and memory in the adult brain.

1.4.2 DNA hydroxymethylation and TETs in the brain

In the adult brain, 5hmC is about 10 times higher than in other organs (Kriaucionis and Heintz, 2009; Li and Liu, 2011) and its abundance is brain-region specific. It is most prevalent in the hippocampus (0.6 % of total cytosine bases), the cortex (0.7 %) and the cerebellum (0.3 %) (Globisch et al., 2010; Kriaucionis and Heintz, 2009; Münzel et al., 2010; Song et al., 2011). 5hmC levels are also cell-type specific and vary based on developmental stage. In cerebellar Purkinje neurons, 0.6 % of cytosines display hydroxymethylation, whereas hydroxymethylation reaches only 0.2 % in granule cells (Kriaucionis and Heintz, 2009). In the frontal cortex, 5hmC content is higher in neurons compared to glia (Lister et al., 2013). Importantly, 5hmC accumulates during brain development in mice (Lister et al., 2013; Song et al., 2011), in particular in developmentally activated genes compared to repressed or silent genes (Szulwach et al., 2011). Similarly, genes

associated with neuronal differentiation acquire 5hmC in the course of differentiation in their bodies. This increase in hydroxymethylation correlates with gene activation (Colquitt et al., 2013; Hahn et al., 2013). Likewise, neuronal activity induces changes in global 5hmC levels in the CA1 region of the hippocampus (Kaas et al., 2013). Overall, 5hmC is subjected to dynamic fluctuations and associated with transcriptional regulation in the brain.

5hmC synthesis is performed by the 5mC dioxygenases, TET1, 2 and 3, all of which require Fe^{2+} as cofactor and alpha-ketoglutarate as cosubstrate (Tahiliani et al., 2009) (Figure 1.2). Although all TET proteins share a conserved C-terminal catalytic domain that includes a double-stranded β-helix fold, they differ in terms of their N-terminal DNA-binding CXXC domain. Both TET1 and TET3 harbour a CXXC domain that binds to CpG dinucleotides. TET2, however, lost its N-terminal CXXC domain, which is now encoded by a neighboring gene termed *Idax*/*Cxx4* (Ko et al., 2013).

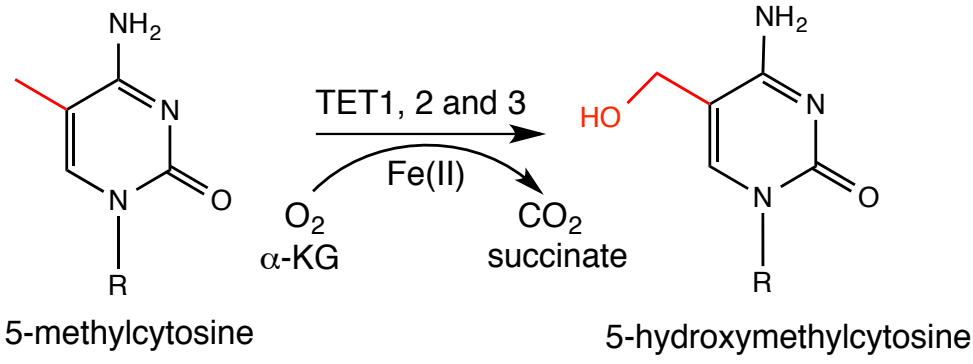

Figure 1-2 Pathway for generation of 5hmC by TET enzymes. TET uses oxygen (O_2) as a substrate to catalyze oxidative decarboxylation of alpha-ketoglutarate (α-KG), thereby generating a reactive high-valent enzyme-bound Fe(IV)-oxo intermediate (not illustrated) that converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). CO_2 and succinate are by-products of the reaction.

TET1, 2 and 3 are all expressed in the nervous system. TET1 is enriched in undifferentiated ESCs and is low in the adult brain, whereas TET2 and 3 are abundant in the adult brain (Szwagierczak et al., 2010). Although all TETs have the same enzymatic activity, they are thought to contribute differently to biological processes due to their different spatial and temporal distribution.

1.4.2.1 TET1

Even though it is only weakly expressed in the adult brain, TET1 is the most thoroughly characterized TET protein to date. TET1 whole body deficiency in mice does not affect brain morphology and development (Rudenko et al., 2013; Zhang et al., 2013b). However, brain processes like neurogenesis and hippocampal long-term depression are altered in these mice (Zhang et al., 2013b). Many studies have reported memory impairment upon *Tet1* KO in mice, but contrasting results have been found as to which memory type and process are affected. Using Morris water maze (MWM), Zhang et al. observed deficits in spatial STM but normal LTM (Zhang et al., 2013b). In contrast, Rudenko and colleagues reported normal memory acquisition but deficits in memory extinction in MWM and fear conditioning (Rudenko et al., 2013). Another group found unaltered memory acquisition in *Tet1* KO mice upon fear conditioning, albeit memory consolidation and LTM was enhanced (Kumar et al., 2015). *Tet1* overexpression in the hippocampus impairs LTM in fear conditioning, whereas STM is not affected (Kaas et al., 2013). This effect is independent of TET1 catalytic activity as inactivation of TET1 catalytic domain leads to similar observations. The contrasting results obtained using the *Tet1* KO model might be due to the fact that all three *Tet* genes are expressed in the brain and may exhibit some functional redundancy.

At the molecular level, Tet1 mRNA is downregulated in response to CFC in dorsal CA1 hippocampus, suggesting that its expression is regulated in an activity-dependent manner (Kaas et al., 2013). *Tet1* overexpression in the hippocampus leads to reduced methylation at the promoters of *Bdnf* and *Fgf1*,

two genes that exhibit activity-induced active DNA demethylation in the dentate gyrus. This effect is accompanied by an upregulation in transcript levels of both *Bdnf* and *Fgf1* (Guo et al., 2011c). Conversely, *Tet1* and *Apobec1* knockdown in the dentate gyrus of the hippocampus prevents activity-induced active DNA demethylation of *Bdnf* and *Fgf1* promoters. This suggests that TET1 and APOBEC1 work synergistically in active DNA demethylation in the mouse brain. Furthermore, *Tet1* overexpression leads to a significant upregulation of several activity-dependent neuronal genes, including *c-Fos*, *Arc*, *Egr1,* as well as genes involved in the DNA demethylation pathway such as *Tdg* and *Apobec1*. Conversely, *c-Fos*, *Arc, Egr2* and *Npas4* expression levels are decreased in the hippocampus of *Tet1* KO mice (Rudenko et al., 2013). Further analysis revealed hypermethylation of the *Npas4* promoter-exon 1 junction region, providing a potential mechanism for the observed downregulation of *Npas4* in *Tet1* KO mice.

1.4.2.2 TET2

Unlike TET1, the role of TET2 in the adult brain has not been extensively studied. TET2 has been proposed to play a role in DNA demethylation of development-dependent genomic regions during brain development. Indeed, whole-genome bisulfite sequencing in the frontal cortex of *Tet2* KO mice identified 4-fold more hypermethylated CpGs compared to wild-type in the course of brain development (Lister et al., 2013). Furthermore, TET2 was found to play a role in synaptic transmission, as shRNA-mediated knockdown of *Tet2* in hippocampal neurons affects basal levels of synaptic transmission (Yu et al., 2015).

1.4.2.3 TET3

Among the TET family, *Tet3* is the most expressed gene in neurons (Colquitt et al., 2013). *Tet3* exists in three major isoforms produced through alternative splicing *i.e.* an oocyte-specific isoform, an isoform containing a CXXC domain,

and an isoform lacking the CXXC domain (Jin et al., 2016). Specifically, the isoform lacking the CXXC domain is enriched in neuronal tissue (Liu, 2013; Perera 2015), and was found to interact with the RE1-Silencing Transcription factor (REST) and various HMTs such as NSD3, NSD2 and SETD2 (Perera et al., 2015). In line with the observation that TET3 is involved in gene transcriptional regulation of neuronal genes, TET3 was proposed to be a transcriptional activator of early eye and neural development in *Xenopus laevis* (Xu et al., 2012).

In summary, epigenetic mechanisms, including DNA (hydroxy)methylation and histone modifications, are implicated in controlling gene expression at the transcriptional level in the brain. The complex network of gene expression and epigenetic regulation is complemented by other regulatory mechanisms. In particular small (19 to 22 nucleotides) endogenous non-coding RNAs termed microRNAs (miRNAs). MiRNAs are potent post-transcriptional regulators that are implicated in the control of numerous biological processes, including memory formation and maintenance (Woldemichael and Mansuy, 2016).

1.5 miRNAs and their regulatory role in the brain

MiRNAs are particularly relevant for the formation of LTM due to their localized and inducible expression level, rapid turnover and combinatorial mode of action. They are therefore ideal candidates for orchestrating and fine-tuning gene expression related to synaptic plasticity and memory formation.

1.5.1 Role of miRNAs in synaptic plasticity and learning and memory

Many miRNAs are enriched in the CNS, mainly in a region-specific manner (Juhila et al., 2011). Interestingly, some miRNAs are specifically enriched in dendrites and synaptosomes, such as miR-134. This miRNA operates locally

to control dendritic spine morphology and development by negatively regulating the expression of the synaptic protein LIMK1 (Schratt et al., 2006). Furthermore, miR-134 overexpression in the hippocampus impairs synaptic plasticity and memory formation via translational repression of *Creb* and *Bdnf* (Gao et al., 2010). Several other studies have reported the importance of miRNAs in regulating gene expression in the context of learning and memory (reviewed in Saab and Mansuy, 2014). In this regard, the best-characterized neuronal miRNAs are miR-132 and miR-212. Deletion of the miR-132/212 locus in excitatory neurons of the hippocampus leads to significant impairments in memory retrieval and spatial memory (Hansen et al., 2016), whereas overexpression of miR-132 enhances cognitive functions (Hansen et al., 2013). Thus, manipulation of specific brain miRNA expression levels affects higher-order brain function *in vivo*.

Activity-dependent regulation of gene expression is crucial for synaptic plasticity and memory formation, and this is also true for many miRNAs. In cultured neurons, miR-132 expression is consistently increased in response to neuronal activity (Klein et al., 2007; Vo et al., 2005; Wayman et al., 2008). Likewise, miR-132 is upregulated in specific brain regions in a variety of learning paradigms, including CFC (Nudelman et al., 2010). CFC induces widespread changes in the hippocampal miRNA profile mostly in an NMDA-dependent manner, suggesting that they are likely related to learning and memory formation (Kye et al., 2011). In line with this finding, numerous studies have shown that the expression of miRNAs is dynamically modulated during neuronal activity and memory formation (reviewed in Sim et al., 2014). In the hippocampus, a fraction of the activity-regulated miRNAs follow a biphasic pattern of expression, i.e., a rapid induction is followed by a decline, whereas other miRNAs undergo no induction but decrease in expression at later time points (Eacker et al., 2011). This is consistent with the observation that miRNAs exhibit a high turnover rate in neurons, and this general property of neuronal miRNAs is dependent on activity (Krol et al., 2010a). Intriguingly, certain miRNAs, such as miR-29b, contain specific sequence elements that

are required for their rapid decay (Zhang et al., 2011). In conclusion, the literature precedent suggests that neuronal-activity dependent regulation of miRNA may fine-tune gene expression patterns that are required for synaptic plasticity and memory formation.

1.5.2 Biogenesis of miRNAs

Mature miRNAs originate from their primary miRNA transcript (pri-miRNA) that is typically transcribed by RNA polymerase II and occasionally by RNA pol III (Borchert et al., 2006; Cai et al., 2004; Lee et al., 2004). Pri-miRNA is composed of a hairpin structure containing a terminal loop, a double-stranded stem and flanking single stranded sequences. The pri-miRNA transcript is cleaved within the nucleus by the microprocessor complex that is composed of DROSHA, a nuclear RNAse III endonuclease, and DiGeorge syndrome critical region gene 8 (DGCR8), a double-stranded RNA binding protein (RBP). Briefly, DGCR8 mediates the recognition of the double stranded stem of the pri-miRNA hairpin, and guides Drosha to the cleavage site. The cleavage site is located at approximately 11 bp away from the basal junction of the stem, and cleavage by DROSHA at this site generates the precursor miRNA (pre-miRNA). Pre-miRNAs are then exported to the cytoplasm via the Exportin-5 pathway (Lund et al., 2004). In the cytoplasm, further processing of pre-miRNAs is carried out by another RNAse III enzyme named DICER. DICER, in combination with its partner TAR RNA binding-protein (TRBP), cleaves the pre-miRNAs at their terminal loops, thus releasing a double stranded RNA of 20-24 nt length. One of these two strands i.e. the guide strand is incorporated into the miRNA-induced silencing complex (miRISC) and becomes functional. The strand selection depends on the thermodynamic properties of each strand. In fact, the strand with the weakest 5' end pairing is preferentially loaded into the miRISC, while the other strand (passenger strand) is released and degraded (Yates et al., 2013). The core of the miRISC is mainly composed of argonaute proteins and their partners GW182 proteins, which play a key role in the assembly and function of miRISCs. The retained miRNA within the miRISC guides the complex to mRNAs. MiRNAs usually

binds to the 3'UTR of target mRNAs through imperfect base-pairing via the seed sequence. This mediates inhibition of protein synthesis by repression of translation. Translational inhibition occurs at both initiation by interfering with ribosome recruitment (Humphreys et al., 2010; Mathonnet et al., 2007) and post-initiation steps (Maroney et al., 2006; Petersen et al., 2006) but the molecular mechanisms involved are still unclear. The miRISC also promotes deadenylation/decapping via recruitment of deadenylase complexes, which facilitate degradation of target mRNAs by exonucleases (Fabian and Sonenberg, 2012) (Figure 1.3). Most miRNAs are produced through the canonical pathway of miRNA biogenesis, involving both transcriptional and post-transcriptional processes. Importantly, the biogenesis of miRNAs is tightly regulated, mainly by RBPs. RBPs are implicated in most steps of miRNA biogenesis i.e. from the production of the pri-miRNA transcript to the miRISC formation (extensively reviewed in Ha and Kim, 2014).

MiRNA loci are located within various genomic contexts with the majority of canonical miRNAs being encoded by introns of coding and non-coding transcripts. Notably, miRNAs are under the control of proximal and distal regulatory regions and their promoters share similar Pol II regulatory elements as those of protein-coding genes, including CGI, TSS, TF binding sites, histone marks, etc (Corcoran et al., 2009; Monteys et al., 2010). Similarly to protein-coding genes, miRNA loci can therefore serve as target for the epigenetic machinery. Conversely, a subset of miRNAs defined as epi-miRNAs can directly target effectors of the epigenetic machinery, such as HDACs and DNMTs.

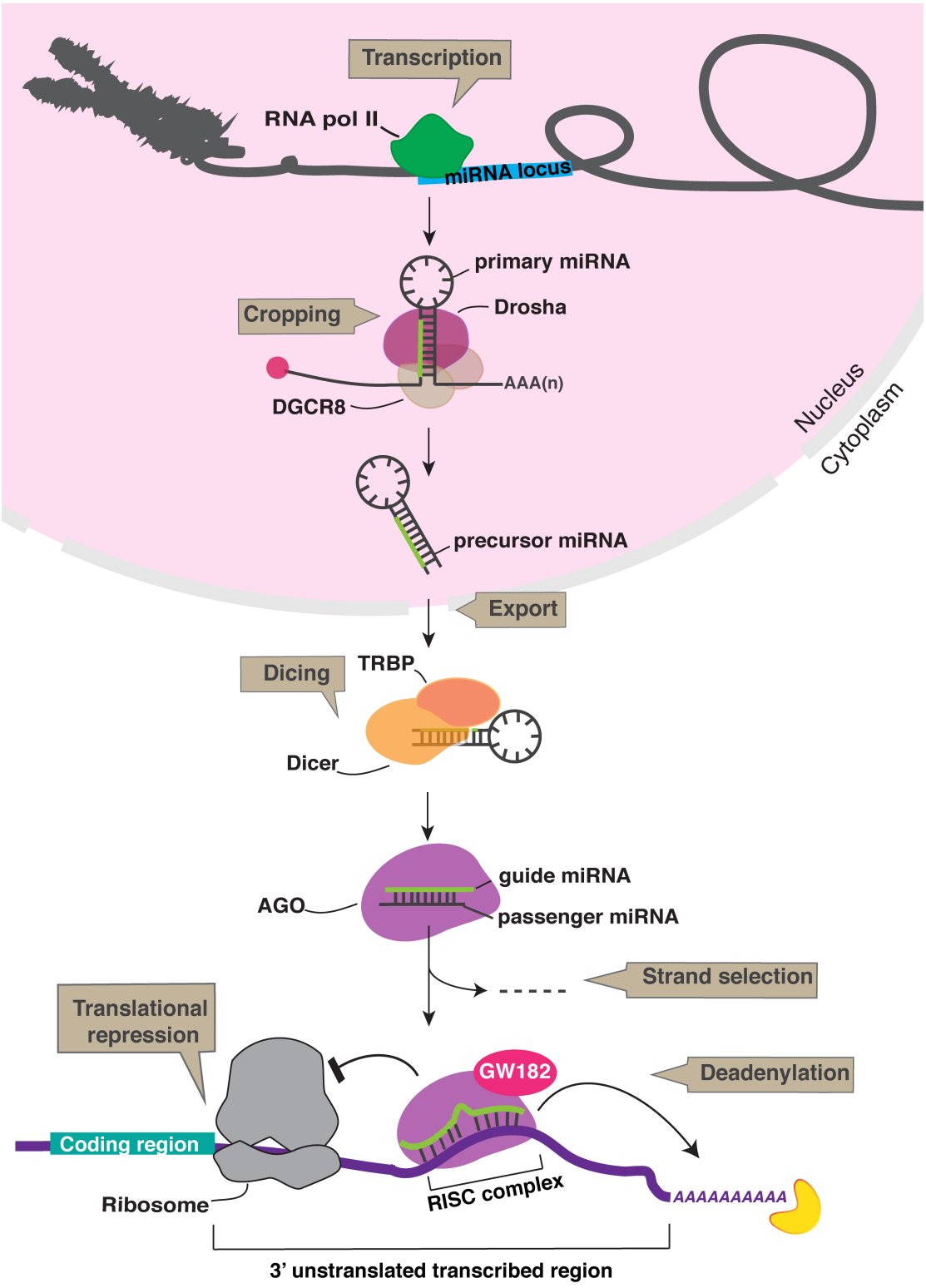

Figure 1-3 Canonical miRNA biogenesis pathway. MiRNAs are transcribed into primary miRNA and processed by the microprocessor, a complex composed of the RNaseIII Drosha, and its binding partner DGCR8. The product of this cleavage, the precursor miRNA, is then exported to the cytoplasm and further cropped by Dicer. The miRNA duplex, composed of the guide miRNA and the passenger miRNA, is loaded onto an AGO protein to form the RISC. After binding to the target mRNA, RISC serves as a scaffold for GW182 and a deadenylase complex that facilitates mRNA degradation.

1.6 Interplay between miRNAs and the epigenetic machinery: epi-miRNAs

The first characterized epi-miRNAs are the members of the miR-29 family. The miR-29 family includes miR-29a, miR-29b-1, miR-29b-2 and miR-29c. MiR-29a and miR-29b-1 form a cluster, and are co-transcribed as a polycistronic primary transcript. Likewise, miR-29b-2 and miR-29c are transcribed together. Mature miR-29s share an identical seed sequence at nucleotide positions 2-7, implying that their target genes highly overlap. Importantly, the miR-29 family has been reported to indirectly influence the DNA methylation machinery by controlling TFs that regulate DNMT1 gene transcription (Garzon et al., 2009) but also by directly targeting *de novo* DNMTs. A study by Fabbri and colleagues revealed that changes in the expression level of miR-29s lead to aberrant patterns of methylation in lung cancer cell lines. In these cells, miR-29s were found to target both DNMT3a and b via direct binding to their 3'UTRs (Fabbri et al., 2007). Interestingly, several *in silico* methods (TargetScan, MiRanda) used for prediction of miRNA-target interactions reported the presence of multiple well-conserved miR29-binding sites within the 3'UTR of all *Tet* transcripts (Figure 1.4).

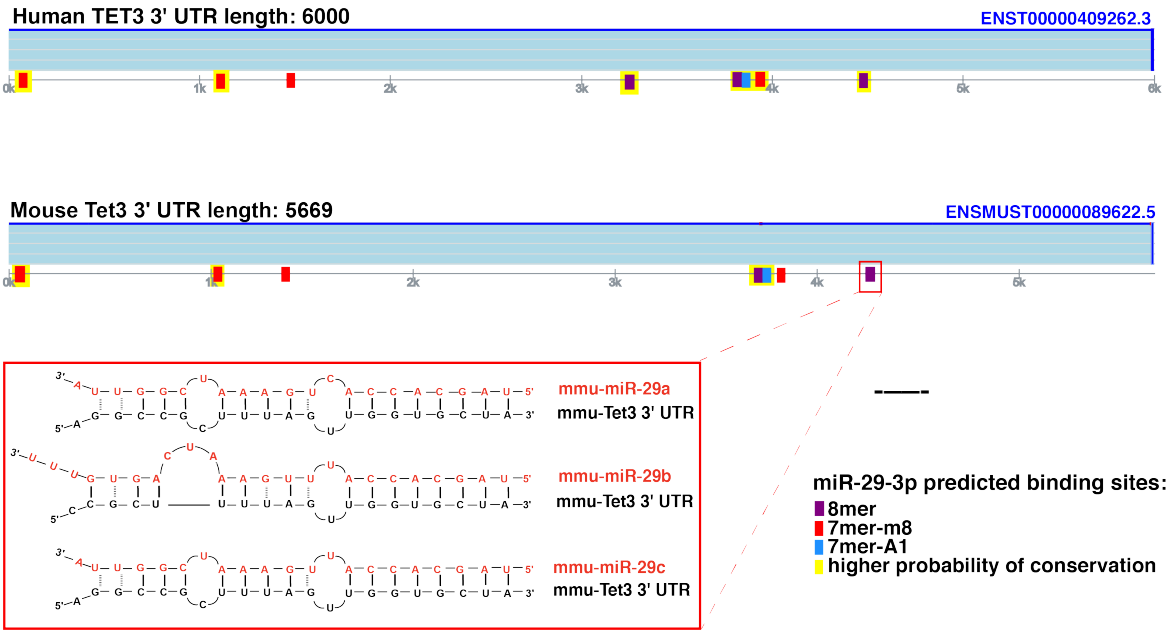

Figure 1-4 miR-29 family has multiple well-conserved and predicted binding sites within *Tet3* 3'UTR. Examples of complementary sites for miR-29s in the 3'UTR region of human and mouse *Tet3* according to TargetScan prediction. Adapted from targetscan.org

This led us to postulate that miR-29s may regulate the expression of both DNA methyltransferases and demethylases. This observation suggests that the expression of miR-29s in the brain likely contribute to the regulation of effectors of the DNA epigenetic machinery, including TET proteins in the context of learning and memory. In the present thesis, we therefore aimed to elucidate the interplay between miR-29s and the TET proteins in the brain upon formation of memory.

1.7 Overview

MiRNAs and epigenetic mechanisms play a central role in neuronal morphogenesis, synaptic plasticity and also in higher-order brain function such as learning and memory (**Chapter 1**). It is therefore unsurprising that disruption of miRNA and epigenetics pathways has been implicated in a broad range of neurodevelopmental, neurodegenerative and psychiatric disorders. In **Chapter 2**, we discuss the contribution of dysregulated epigenetic mechanisms to the etiology and pathophysiology of neurodevelopmental and neurodegenerative disorders, using Rett syndrome and Alzheimer's disease as examples. Further, it describes the involvement of epigenetic mechanisms in the development of depression and their role in mediating the effect of anti-depressants, illustrating the role of the epigenome in psychiatric disorders. It ends with a brief description of how epigenetic modifications induced by early life trauma can influence brain functions in the adult, and how these effects are thought to be transmitted through epigenetic mechanisms across generations.

In the brain, active DNA methylation and demethylation are dynamic processes that are required for learning and memory formation. DNMTs are induced in the brain after learning, but the mode of regulation of TETs remain mostly unexplored. In **Chapter 3**, we characterize the activity-dependent expression of TET family members both *in vivo* and *in vitro*, and further investigate the possibility that they are under the control of miR-29-mediated

regulatory mechanisms. The findings of the thesis are summarized and further discussed in **Chapter 4**. In this chapter, we also conclude by proposing a model by which TETs are regulated in the brain upon neuronal activity.

2 Epigenetics of Brain Disorders

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2.1 Abstract

Emerging evidence suggests that epigenetic mechanisms are necessary for the development and maintenance of neuronal networks in the brain, and for higher-order brain processes like cognitive functions and behavior. Defects in epigenetic mechanisms are now known to alter disease susceptibility, contribute to the etiology or pathophysiology of some disorders, and also determine the response to therapies. A large body of literature implicates epigenetic dysfunctions in neurodevelopmental disorders such as Rett syndrome (RS), Fragile X and Rubinstein-Taybi syndrome, and in neurodegenerative and psychiatric conditions like Alzheimer disease (AD), frontotemporal lobar degeneration (FTLD), depression and schizophrenia. Epigenetic mechanisms also contribute to transgenerational effects of the environment on brain and body functions, and to the resulting inheritance of pathologies across families.

2.2 Introduction

The brain is a complex organ that is highly plastic during development and that keeps some plasticity and responsiveness throughout life. In mammals, this plasticity is particularly high during pre-natal, post-natal and pubertal periods. It is characterized by the formation and maintenance of synapses, the formation of new neurons, their integration into existing neuronal networks, and the re-organization of neuronal networks. This plasticity is essential for brain processes such as learning and memory formation, and requires active changes in gene expression. Several brain disorders result from dysfunctional or impaired regulation of the molecular processes governing brain plasticity, underscoring their potential role in the pathogenesis of brain disorders.

Gene expression in the brain is modulated in a cell, region, and context-specific manner by epigenetic mechanisms. These mechanisms operate at the transcriptional and post-transcriptional level in response to activity. They can implicate several processes involving DNA methylation (reviewed in (Tognini et al., 2015)) histone modifications (reviewed in (Rudenko and Tsai, 2014)), and non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) (reviewed in (Follert et al., 2014; Wang et al., 2012)). Additional processes like prion-like seeding (reviewed in (March et al., 2016)), nucleosome positioning (Brown et al., 2015), histone turnover (Maze et al., 2015), competitive endogenous RNAs (reviewed in (Thomson and Dinger, 2016)) also constitute active non-genetic regulation in the brain. This chapter describes the contribution of DNA methylation, histone modifications, and miRNAs to brain disorders using a neurodevelopmental, neurodegenerative and psychiatric disorders as examples. It also indicates how epigenetic processes contribute to the effects of early post-natal life adversity on brain functions and their transmission to subsequent generations.

2.2.1 Epigenetic mechanisms important for the brain

2.2.1.1 DNA methylation

DNA methylation most commonly occurs at cytosine-guanine dinucleotides (CpG), although CpH methylation (H is A, C or T) is also present in the human and mouse brain postnatally (Guo et al., 2014; Lister et al., 2013). CpG methylation has traditionally been viewed as a fairly stable epigenetic mark mostly responsible for gene silencing. Silencing takes place by direct inhibition of the binding of transcription factors, or by recruitment of methyl-CpG binding proteins (MBPs) and associated repressive chromatin-remodeling components (Bird, 2002; Klose and Bird, 2006). However since CpG methylation is also present at the promoter and coding region of actively transcribed genes, it can also be associated with transcriptional activity (Bahar Halpern et al., 2014; Jones et al., 1998). Recent evidence has shown that in many cells including post-mitotic cells like adult neurons, DNA methylation is dynamically regulated and CpGs can be actively methylated and demethylated. DNA demethylation involves a succession of biochemical steps implicating several enzymes. During this process, 5-methylcytosine (5mC) is successively oxidized into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5cAC) by ten-eleven translocation (TET) proteins. 5fC and 5cAC can ultimately be removed and repaired by terminal deoxytidyl transferases generating an unmodified cytosine (Kohli and Zhang, 2013). In contrast to 5fC and 5cAC, 5hmC is enriched in the adult brain, especially in the hippocampus, cortex and cerebellum (Globisch et al., 2010; Münzel et al., 2010; Song et al., 2011). The high abundance and stability of 5hmC in the brain (Bachman et al., 2014), suggest that this cytosine modification may not only be a transient by-product of 5mC metabolism but may also serve as an independent epigenetic mark thought to play an important role in brain processes. As both DNA methylation and hydroxymethylation patterns are modulated during development and upon neuronal activity and memory processes in different brain regions (Lister and

Mukamel, 2015), alterations in their profile may be relevant for the development of brain disorders.

2.2.1.2 Histone modifications

Covalent posttranslational modifications (PTMs) of histone proteins are other important epigenetic marks that primarily involve acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation (reviewed in (Bannister and Kouzarides, 2011)). These modifications control the accessibility of the chromatin to the transcriptional machinery, in a sequence and activity-dependent manner (Bannister and Kouzarides, 2011). Generally, acetylation and phosphorylation, occurring respectively on lysine and serine, threonine or tyrosine (residues, are associated with transcriptional activation (Li et al., 2007). Histone methylation on lysine is associated with both actively transcribed and silenced genes (Klose and Zhang, 2007; Peters and Schübeler, 2005). Similarly, histone ubiquitination is associated with both transcriptional silencing and activation depending on whether it occurs on H2A or H2B respectively (reviewed in (Cao and Yan, 2012)). Finally, histone SUMOylation, which requires addition of small-ubiquitin like modifier (SUMO) to histones, negatively regulates gene transcription in the brain, often in association with other epigenetic regulators (Cheng et al., 2014a; Stielow et al., 2014). Importantly, histone PTMs are integral to the regulation of important physiological brain processes. For instance, synaptic mechanisms underlying hippocampal memory formation require dynamic changes in histone acetylation and methylation (Gräff et al., 2012a; Kerimoglu et al., 2013; Mahgoub and Monteggia, 2014). Similarly, histone acetylation is essential to the resistance of neurons against ischemic or oxidative insult (Ryu et al., 2003; Yildirim et al., 2014). Enzymes important for regulation of major histone PTMs, such as histone acetyl transferases (HATs), histone deacetylases (HDACs), and histone methyltransferases (HMTs) are abundant in the brain. Owing to this and the important roles for histone PTMs in brain

processes, their critical involvement in brain disorders has become an important topic of current investigations (Volmar and Wahlestedt, 2015).

2.2.1.3 MicroRNAs

Epigenetic regulation of gene expression can also be achieved by ncRNAs like miRNAs. MiRNAs are 20-22 nucleotide long RNAs that act by translational repression or degradation of mRNA targets (reviewed in (Ha and Kim, 2014)). MiRNA biogenesis involves a succession of steps in the cell regulated by the RNAses Drosha and its partner DiGeorge syndrome critical region 8 (DGCR8) in the nucleus, followed by further processing by another RNAse Dicer and its partner TAR RNA binding protein (TRBP) in the cytoplasm. Once formed, miRNAs associate with Argonaute proteins in the cytoplasm to form an RNA-induced silencing complex (RISC). A number of RNA-binding proteins can additionally modulate the efficiency of the microprocessor, Dicer or RISC (reviewed in (Ha and Kim, 2014)). Many miRNAs and components of miRNA biogenesis machinery are abundantly expressed in the brain, mostly in a region-specific manner. They have been linked to important brain processes like neurogenesis (reviewed in (Wakabayashi et al., 2014)), neuronal activity (reviewed in (Elramah et al., 2014)), and memory formation (reviewed in (Saab and Mansuy, 2014)). Animal models with experimental manipulation of global miRNA biogenesis or functional inhibition/overexpression of specific miRNAs can lead to pathological phenotypes, highlighting their important contribution to brain disorders (reviewed in (Wang et al., 2012)). Importantly, some proteins like the methyl-DNA binding protein MeCP2 or the protein phosphatase PP1 can regulate multiple epigenetic pathways in a tissue- and context-specific manner (Gräff and Mansuy, 2008; Jenuwein and Allis, 2001; Turner, 2002). When the expression of such epigenetic regulators is perturbed, for instance experimentally and in disease states, many core brain processes are affected, underscoring their likely implication in brain disorders (Gräff and Mansuy, 2008; Jenuwein and Allis, 2001; Turner, 2002).

2.2.2 Epigenetic dysregulation in neurodevelopmental disorders - The example of Rett syndrome

Neurodevelopmental disorders are characterized by impaired functions of the central nervous system (CNS) that appear early in development and often persist into adulthood. The impairments may manifest early in life like in case of fetal alcohol syndrome, or later. Several genetically-determined neurodevelopmental disorders have been documented to also involve epigenetic dysregulation that alter physiological functions (reviewed in (Millan, 2013)). This chapter focuses on Rett syndrome (RS), a disorder associated with aberrant DNA methylation, histone PTMs and miRNAs (reviewed in (Kubota et al., 2013; Lyst and Bird, 2015)).

RS is a relatively common (worldwide prevalence of 1:10'000) and progressive neurological disorder characterized by an arrest of CNS development and intellectual disability. It is generally caused by loss-of-function mutations in the X-linked *methyl-CpG-binding protein 2* (*MeCP2*) gene (Amir et al., 1999), which is lethal when hemizygous in males, and therefore affects exclusively females. MeCP2 is normally abundant in the brain, in particular in neurons where its level is comparable to that of histone octamers (Skene et al., 2010). It is a member of the methyl binding protein (MBP) family that binds methylated CpGs with high affinity to regulate gene transcription in a bidirectional fashion. MeCP2 has three domains, a methyl binding domain (MBD), a transcriptional repressor domain (TRD), and a WW domain (Weaving et al., 2005). MeCP2 not only binds to methylated CpG, it also recognizes methylated CpH (H=A, C or T) (Guo et al., 2014), and hydroxymethylated CpGs (Mellen et al., 2012), suggesting that it substantially decorates DNA and when deficient, like in *MeCP2* mutant mice, may strongly perturb the profile of 5mC and 5hmC (Szulwach et al., 2011). This can have significant functional consequences since MeCP2 binding to 5mC contributes to transcriptional regulation through the recruitment of HDACs and other

transcriptional co-repressor complexes, such as Sin3a/HDACI, resulting in chromatin compaction and gene silencing (Weaving et al., 2005). In addition to acting as a transcriptional repression, MeCP2 is thought to also function as transcriptional activator. This may be by interacting with transcription factors like CREB1 at the promoter of target genes (Chahrour et al., 2008), and/or through binding to 5hmC, an epigenetic mark enriched in the body of highly expressed genes. Hence, MeCP2 appears to be an adapter molecule, acting as a promiscuous DNA binding protein that helps recruits transcriptional regulators.

In humans, RS can be caused by loss-of-function mutations in either *MeCP2* MBD or TRD (Amir et al., 1999). Mis-sense mutations are generally milder while non-sense mutations cause more severe symptoms. Similarly, MBD mutations generally lead to a more severe phenotype (Weaving et al., 2005). In mice, neuron-specific *MeCP2* deletion has been shown to recapitulate RS symptoms. MeCP2-deficient mice have reduced brain weight and abnormal brain circuitry (Chen et al., 2001), an overall decrease in exploratory activity (Chen et al., 2001; Guy et al., 2001), cognitive deficits and impaired synaptic plasticity (Gemelli et al., 2006). MeCP2 ablation in forebrain GABAergic neurons also impairs sensory information processing (Goffin et al., 2014), a symptom present in RS patients. Some of the cognitive deficits induced by MeCP2 deficiency could be reversed by overexpression of wild-type human MeCP2 in young mutant mice (Collins et al., 2004), confirming that they resulted from a lack of MeCP2. However at the same time, MeCP2 overexpression induces seizures, suggesting that excess in MeCP2 is also deleterious for brain functions, and may lead to neurological functions related to RS.

Since MeCP2 functions as a transcriptional regulator, dysregulation of its target genes likely contribute to RS symptoms. Global transcriptome analyses in human RS brain and MeCP2-deficient mice have shown dysregulation of a multitude of genes. Genes with long sequences, in particular, appear to be

prime targets of MeCP2-mediated transcriptional repression in the mouse brain (Gabel et al., 2015; Sugino et al., 2014). Many of these long genes encode proteins implicated in neuronal physiology, axon guidance and synapse formation such as the calcium/calmodulin-dependent kinase *Camk2d*, and the voltage-gated potassium channel *Kcnh7*, and may explain neuronal dysfunctions in RS (Gabel et al., 2015). Importantly, these genes contain a high density of methylated CpA required for length-dependent gene repression by MeCP2.

An important target is the brain-derived neurotrophic factor (*Bdnf*) gene, which expression is reduced in *MeCP2-null* mice. Overexpression of BDNF in *MeCP2-null* mice ameliorates the phenotype, it reduces neuronal atrophy and improves survival (Chang et al., 2006). In addition, delivery of exogenous BDNF restores synaptic dysfunctions in *MeCP2-null* mice (Kline et al., 2010), suggesting that changes in gene expression in MeCP2 mutants have an impact on RS phenotype. Further to *Bdnf*, many other genes including myelin-associated proteins and dopamine decarboxylase, have been identified as direct binding targets of MeCP2 in the mouse brain (Gabel et al., 2015; Urdinguio et al., 2008). MeCP2 also interacts with other proteins, including chromatin-modifying factors (Jones et al., 1998; Nan et al., 1998), epigenetic regulators such DNMT1 and TET1 (Cartron et al., 2013), and transcriptional modulators, such as coREST, suv39H1, cSK1, etc. (Lyst and Bird, 2015), and therefore can influence the epigenetic profile (DNA methylation) of target genes. It can also influence histone PTMs, in particular histone acetylation and methylation (see Figure 2.1).

For *Bdnf*, these changes are mediated by the formation of a complex between MeCP2 and HDAC1, reducing H3 and H4 acetylation. This is paralleled by increased H3K9 dimethylation, which inhibits gene transcription, but decreased H3K4 dimethylation, which promotes gene transcription (Kouzarides, 2007).

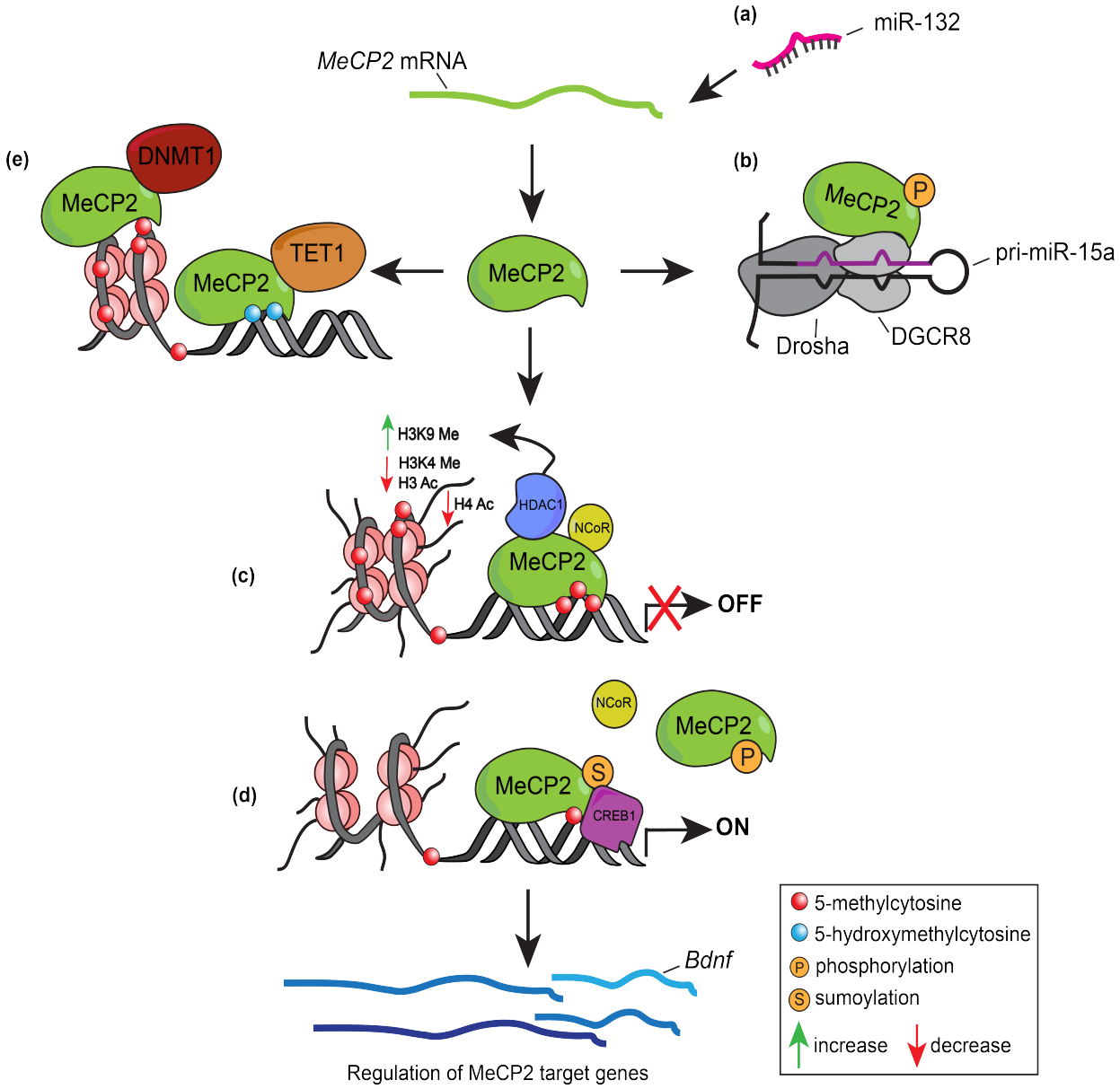

Figure 2-1 Roles of MeCP2 as regulator of the epigenetic code. (**a**) MeCP2 translation is regulated by miRNAs. For example, miR-132 targets MeCP2 mRNA in neurons, which ultimately leads to increased *Bdnf* transcript levels. (**b**). MeCP2 regulates gene expression post-transcriptionally by modulating miRNA processing via interfering with the assembly of Drosha and DGCR8 complex. MeCP2 can selectively modulate the biogenesis of certain miRNAs, in particular miR-15a. This miRNA is implicated in neuronal maturation by controlling *Bdnf* expression levels. (**c**) MeCP2 functions as a transcriptional repressor by either recruiting transcriptional co-repressor complexes (e.g NCoR) and/or histone deacetylases (e.g HDAC1). Recruitment of HDAC1 results in reduced acetylation of H3 and H4 at *Bdnf* promoter as well as increased H3K9 dimethylation and decreased H3K4 dimethylation. This leads to chromatin condensation, thus limited access to

the transcriptional machinery and mediates decreased *Bdnf* transcription. (**d**) MeCP2 also functions as a transcriptional activator by interacting with transcription factors such as CREB1 at the promoter of target genes. Recruitment of CREB1 results in chromatin remodeling permissible for transcription. In addition, phosphorylation of MeCP2 can result in the inability of MeCP2 to bind to its methylated binding sites and interferes with the recruitment of NCoR. Further, MeCP2 SUMOylation increases CREB DNA binding, thus enhancing *Bdnf* mRNA expression. (**e**) MeCP2 binds to both methylated and hydroxymethylated cytosines, and interacts with epigenetic regulators such as DNMT1 and TET1. MeCP2 could therefore influence the epigenetic profile (DNA methylation) of target genes such as *Bdnf*.

This indicates complex and possibly self-compensatory mechanisms between transcriptional programs regulated by MeCP2 as a result of its dual role in DNA methylation and histone modifications (Kubota et al., 2013; Shahbazian et al., 2002). Notably, the activity and expression of MeCP2 itself is under the control of posttranslational modifications. Activity-dependent phosphorylation of MeCP2 at threonine 308 regulates its transcriptional repressor activity by modulating its interaction with the nuclear receptor co-repressor complex (NCoR) (Ebert et al., 2013). Similarly, MeCP2 SUMOylation is an important regulator of transcriptional repressor activity which can rescue the behavioral deficits in mutant-MeCP2 model of RS (Tai et al., 2016).

Further to DNA methylation and histone modifications, miRNAs are also involved in RS symptoms (Lyst and Bird, 2015). Some miRNAs mediate their effect by regulating MeCP2 expression, for instance, MeCP2 is a putative target of miR-132, a CREB-dependent miRNA (Klein et al., 2007). Similarly, miR-22 promotes the differentiation of smooth muscle cells from stem cells by controlling post-transcriptional expression of MeCP2 (Zhao et al., 2015). MiRNAs may also be down-stream effectors of MeCP2 dysfunction in RS since MeCP2 was recently shown to regulate the microprocessor-mediated biogenesis of miRNAs by interfering with Drosha-DGCR8 binding (Cheng et al., 2014b). Through this interaction and possibly others, MeCP2 can selectively modulate the microprocessor-mediated biogenesis of miR-137

(Smrt et al., 2010), miR-199a (Tsujimura et al., 2015), and miR-15a (Gao et al., 2015). These miRNAs regulate important molecular pathways, such as neuronal maturation (Smrt et al., 2010), mammalian targets of rapamycin signaling (Tsujimura et al., 2015), and BDNF expression (Gao et al., 2015) respectively, and hence likely contribute to RS etiology and/or phenotype.

In conclusion, a large body of evidence supports the involvement of epigenetic mechanisms in RS, primarily related to a multi-dimensional and intricate role of MeCP2 as regulator of the epigenetic code. Future studies should examine the potential crosstalk of different epigenetic mechanisms regulated by MeCP2 to identify safe and potent therapeutic targets for RS.

2.2.3 Epigenetic dysregulation in neurodegenerative disorders - The example of Alzheimer's disease

Neurodegenerative diseases are pathological conditions characterized by a gradual loss of cells in the nervous system. In the CNS, such loss usually has devastating consequences on cognition and locomotion. A hallmark feature of most neurodegenerative conditions is intra-cellular or extra-cellular deposition of pathologically aggregated proteins. These pathological deposits include beta-amyloid (β-amyloid) in the case of Alzheimer disease (AD), Tau in the case of AD and frontotemporal lobar degeneration (FTLD), TAR DNA binding protein of 43 kDa (TDP-43) in the case of amyotrophic lateral sclerosis, FTLD, fused in sarcoma in the case of FTLD, and Huntingtin in the case of Huntingtin's disease. Emerging evidence suggests that epigenetic mechanisms contribute to the deposition of these pathological aggregates, and to pathways leading to neuronal death and/or cognitive and motor dysfunction downstream of the pathological deposits (reviewed in (Jakovcevski and Akbarian, 2012; Landgrave-Gómez et al., 2015)). Here, AD is used as a prototype neurodegenerative condition to discuss the contribution of different epigenetic mechanisms in brain degeneration.

AD is one of the most common neurodegenerative diseases worldwide with an estimated prevalence of 5-7% above the age of 60 years in most world regions (Prince et al., 2013). AD pathology is characterized by two major hallmarks in the brain: extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs) (LaFerla and Kitazawa, 2005). Amyloid plaques are deposits of the amyloid β (Aβ) peptide, produced through enzymatic cleavage of the amyloid precursor protein (APP) by β and γ secretases. NFTs are intraneuronal aggregates of hyperphosphorylated tau, a microtubule-binding protein (reviewed in (Serrano-Pozo et al., 2011)). E4 polymorphism of apolipoprotein E, a cholesterol carrier protein with additional roles in Ab metabolism and transport across the blood brain barrier, is the largest known genetic risk factor for late-onset AD (75).

Epigenetic mechanisms have been extensively studied in the CdK-p25 mouse model of AD. Cyclin dependent kinase 5 (Cdk5) and its regulatory subunit p35 are important for CNS development. p25, a truncated form of p35, increases Cdk5 activity, leading to tau hyperphosphorylation, formation of NFTs, astrogliosis and neurodegeneration (Patrick et al., 1999). Studies on CdK-p25 AD model implicated altered histone PTMs in AD (78-81).

Histone PTMs, especially aberrant histone acetylation may contribute to AD through an interaction of the components of APP-Aβ pathway and histone acetylation machinery in neurons. Both hyper and hypoacetylation have been reported in AD. Hyperacetylation is in part related to APP-Ab dependent regulation of HATs. APP intracellular domain, which is produced from APP by γ secretase, forms a complex with the nuclear adaptor protein Fe65 and the HAT TIP60 to activate transcription of genes (Cao and Südhof, 2001). Further, *presenilin 1 (PS1)*, a gene coding for the γ secretase complex, may itself contribute to histone hyperacetylation in AD pathology. Loss-of-function mutations in *PS1* or mutations associated with familial AD inhibit the proteasomal degradation of the HAT CREB binding protein (CBP), and result in increased CREB-mediated gene expression in cultured neurons

(Marambaud et al., 2003). Consistent with the involvement of hyperacetylation, overexpression of the HDAC SIRT1 (silent mating type information regulation 2 homolog 1) in CdK-p25 mice confers substantial protection against AD-related pathologies and memory loss (Kim et al., 2007).

In contrast, other lines of evidence suggest that AD is associated with histone hypoacetylation related to a decreased activity of HATs or an increase in HDACs. In cultured cortical neurons, the overexpression of APP decreases H3 and H4 acetylation by reducing the level of CBP (Rouaux et al., 2003). Similarly, loss-of-function mutations in *PS1* and *PS2* genes in mice reduce the expression of CBP, as well as CBP/CREB target genes *c-fos* and *BDNF* (Saura et al., 2004). Further, HDAC2 is significantly increased in hippocampal CA1 neurons in an AD mouse model, which correlated with decreased promoter acetylation and expression of many plasticity related genes. Virus-mediated knock-down of HDAC2 increases the expression of these genes, and improves cognitive functions in these mice, indicating that HDAC2 induces an epigenetic blockade on cognitive functions in AD. This blockade can be induced not only by Ab oligomers but also by other neurotoxic stimuli, such as hydrogen peroxide, and depends on the binding of stress elements to HDAC2 promoter (Gräff et al., 2012b). Moreover in CdK-p25 AD mice, the intracerebroventricular injection of sodium butyrate, a potent class I/II HDAC inhibitor, rescues memory loss and reinstates synaptic connectivity (Fischer et al., 2007). Components of APP-Ab pathway can themselves be regulated by histone PTMs. For example, cellular models of AD show increased histone acetylation of PS1 and BACE1, and increased expression of HAT p300 (Lu et al., 2014). Similarly, apolipoprotein E4, which increases the risk of AD, favors the nuclear translocation of histones in human neurons, and reduces BDNF expression through low-density lipoprotein receptor related protein (Sen et al., 2015).

Changes in histone in AD are not limited to acetylation but also include altered methylation and ubiquitination, as observed in the frontal cortex of AD patients

(Anderson and Turko, 2015). These changes may contribute to AD by regulating Tau deposition, or by mediating neurotoxicity downstream to pathological Tau aggregation. Phosphorylation and acetylation of Tau inhibit its physiological binding and stabilization of neuronal microtubules, and instead promote tau insolubility and aggregation (Cohen et al., 2011; Gong et al., 2000). Further, mimic of Tau Lys 280 acetylation exacerbates human Tau-mediated neurotoxicity in Drosophila (Gorsky et al., 2016). In contrast, histone lysine methylation occurs endogenously in normal human brains, and resists tau aggregation *in vitro* (Funk et al., 2014). Interestingly, a recent study showed that Tau protein has intrinsic HAT activity, and can self-acetylate (Cohen et al., 2013). This observation may explain why overexpressing Tau leads to Tau pathological aggregation, and questions the utility of HDAC inhibitors in Tau models of AD and other taupathies.

Further to histone PTMs, DNA methylation and hydroxymethylation might also be involved in the pathology of AD. In general, global levels of DNMT and TET enzymes in AD brain regions, and the presence of their reaction products, 5mC and 5hmC, have been investigated in several studies. AD patients have decreased levels of 5mC and DNMT1 in neurons of the entorhinal cortex (Mastroeni et al., 2010). Similarly, a global reduction of 5mC and 5hmC is observed in the hippocampus of AD patients (Chouliaras et al., 2013). Another study however, reported opposite results with increased level of 5mC, 5hmC and TET1 in hippocampal tissue (Bradley-Whitman and Lovell, 2013). In the frontal and temporal cortex of AD patients, both 5mC and 5hmC are elevated (Coppieters et al., 2014). Further analyses are therefore required to understand these discrepancies. Gene-specific alterations in DNA methylation have also been identified in AD patients. A *postmortem* study in humans reported hypomethylation of the presenilin promoter region in late-onset AD patients when compared to age-matched healthy subjects (Wang et al., 2008). Furthermore, *in vitro* hypomethylation of the promoter region of *PS1* increases presenilin expression, which enhances β-amyloid formation (Scarpa et al., 2003). This effect can be reversed by application of the methyl donor S-

adenosylmethionine (SAM) that rescues methylation, decreases presenilin expression and reduces β-amyloid formation. These observations suggest that methyl donors or drugs targeting the methyl metabolism may be potential therapeutic agents to treat AD (Scarpa et al., 2006).

In contrast, other AD-related susceptibility genes such as *BACE1*, which codes for β-secretase, or the gene coding for apolipoprotein E are hypermethylated in late-onset AD (Wang et al., 2008). This suggests that DNA methylation is presumably altered bidirectionally and in a gene-specific manner in AD, similar to histone acetylation. Recently, two independent epigenome-wide association studies identified genome-wide DNA methylation profiles from brain tissue of patients with AD (De Jager et al., 2014; Lunnon et al., 2014). These studies uncovered site-specific methylation alterations and expression changes in genes not previously associated with AD. In particular, four novel loci were independently identified, underpinning their likely association with AD risk. Furthermore, AD-associated genes and genes important for neuronal functions are differentially hydroxymethylated in the hippocampus of a mouse model of AD as demonstrated via 5-hmC genome-wide profiling (Shu et al., 2016). This study further showed that treatment of culture hippocampal neurons with toxic Aβ (1-42) peptide decreases global 5-hmC expression. Further studies are, however, needed to identify the alterations in DNA methylation and hydroxymethylation at specific genes and their possible effect on gene expression to better characterize the mechanisms underlying AD pathogenesis.

MiRNAs may contribute to AD pathology by post-transcriptionally regulating the expression of proteins involved in the production or clearance of amyloid-b. For example, BACE1 expression can be regulated by miR-195 (Zhu et al., 2012), miR-188 (Zhang et al., 2014), and miR-339-5p (Long et al., 2014). Similarly, miR-153 suppresses the expression of APP (Long et al., 2012). In addition to amyloid production, proteins important for amyloid clearance across the blood-brain barrier, such as receptor for advanced glycosylation

end products can also be regulated by miRNAs (Mercado-Pimentel et al., 2015). Further, miRNAs are also involved in the production of Tau, the major component of neurofibrillary tangles. Tau expression is under the control of miR- 219 (Santa-Maria et al., 2015). Similarly, miR-125b (Banzhaf-Strathmann et al., 2014), miR-26b (Absalon et al., 2013), and miR-922 (Zhao et al., 2014) induce Tau phosphorylation by regulating Tau-related kinases and phosphatases, which correlates with cognitive deficits in AD models. Finally, miRNAs may link amyloid/tau deposition to pathways responsible for neuronal death and/or cognitive deficits in AD. Cognitive deficits in an AD transgenic model were related to miR-206-dependent dysregulation of BDNF (Lee et al., 2012). Similarly, another study showed that oxidative damage produced by soluble Ab peptide is likely mediated by altered expression of miR-145 and miR-210 (Li et al., 2014a). Finally, miR-146a arbitrates the inflammatory circuits in AD models and stressed human brain cells through nuclear factor kappa-light-chain-enhancer of activated B cells NFkB (Lukiw et al., 2008).

In conclusion, it is becoming increasingly clear that epigenetic processes are implicated in AD at multiple levels. They mediate the effects of AD risk factors, regulate the expression of many proteins involved in amyloid production and/or clearance, block cognitive functioning in AD, and are responsible for neuronal death downstream from pathological Aβ/Tau aggregation. Some epigenetic marks like histone acetylation are currently being investigated as biomarkers in AD (Wey et al., 2016) and HDAC inhibitors for potential therapeutic approaches. However, the widely used non-specific HDAC inhibitor valproic acid could not reverse cognitive decline or neuropsychiatric symptoms associated with AD in a double-blind place-controlled trial (Tariot et al., 2011). More selective inhibitors may have better outcomes (Falkenberg and Johnstone, 2014). The involvement of epigenetic mechanisms in mediating the risk of AD also highlights their implication in AD prevention, particularly in selected groups. Of particular note here are dietary factors, environment enrichment, and exercise, as they all have potential to change

gene expression epigenetically, and have been shown to delay/ameliorate the symptoms of AD. Emerging evidence suggests that certain diets and exercise can have protective effects in AD through possible epigenetic regulation of BDNF. Similarly, polyphenols in diet have the potential to counteract miRNAs alterations induced by apolipoprotein E knock-out, indicating their potential for delaying AD in individuals with apolipoprotein E4 polymorphism (Dauncey, 2013, 2014, 2015).

2.2.4 Epigenetic dysregulation in psychiatric disorders - The example of depression

Psychiatric diseases like depression and personality, anxiety or psychotic disorders are multi-faceted conditions with complex etiology, and are often difficult to treat. A substantial body of evidence suggests that depressive phenotypes are partly caused by epigenetic mechanisms (see Figure 2.2).

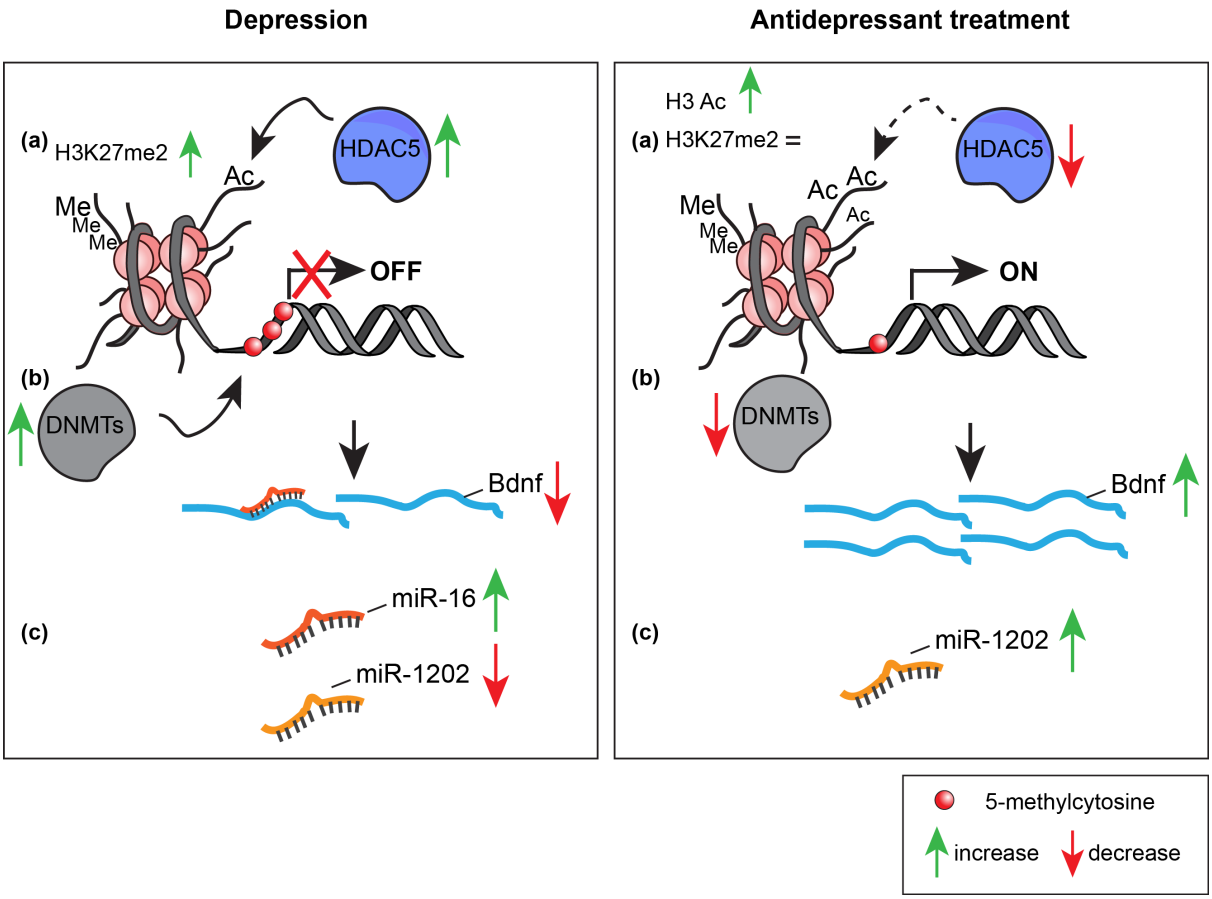

Figure 2-2 The importance of posttranslational histone modifications (PTMs), DNA methylation and miRNAs in depression. (**a**) Alterations in histone PTMs. Upon chronic social defeat stress, a rodent model of depression, H3K27 dimethylation is increased at *Bdnf* promoter. This leads to chromatin condensation, which shuts down *Bdnf* gene expression. In parallel, global H3 acetylation levels are decreased, accompanied by increased HDAC5 expression. Chronic antidepressant (imipramine) treatment reduces HDAC5 levels, leading to increased H3 acetylation, whereas H3K27 dimethylation remains unaffected. Nonetheless, the increase in H3 acetylation is sufficient to reinstate *Bdnf* gene expression. (**b**) Alterations in DNA methylation. Depression in humans is associated with increased DNMT expression in the brain and differential methylation levels at several candidate gene promoters such as *Bdnf*. The increase in 5-methylcytosine levels at the *Bdnf* promoter correlates with low *Bdnf* expression in patients suffering from depression. Antidepressant treatment of blood cells from depressed individuals (paroxetine) results in reduced DNMT1 activity, decreased *Bdnf* methylation, which leads to increased *Bdnf* expression. (**c**) Alterations in miRNA profiles. Depression-like behaviors in rats induced by maternal

deprivation are associated with increased miR-16 expression, a miRNA that targets *Bdnf*, in the hippocampus, as well as, reduced *Bdnf* transcripts. In the blood of individuals with depression, the level of miR-1202 is reduced, while antidepressant treatment reverses this effect.

According to DSM-V, major depressive disorder (MDD) is characterized by low mood, markedly diminished interest or pleasure in activities, fatigue or lethargy, feelings of worthlessness or excessive guilt, psychomotor agitation or retardation, and abnormal weight or sleep changes (American Psychiatric Association). This chronic illness affects a sizable population with about 1.5% to 19% life-time prevalence worldwide (Kessler and Bromet, 2013; Weissman et al., 1996). Depression is difficult to treat, and only half of depressed patients show complete remission (Berton and Nestler, 2006; Tsankova et al., 2007). A major issue with most treatments is that the symptoms are usually ameliorated only after a few weeks of therapy. The reasons for such delay are not entirely known but could reflect the contribution of epigenetic mechanisms to the etiology of depression. Epigenetic mechanisms are also implicated in the appearance of depression after stress exposure, and in the effect of anti-depressants (Menke and Binder, 2014).

The importance of histone PTMs in depression can be appreciated from a rodent model of social defeat. This model of chronic stress induces symptoms of depression that can be reversed with long-term antidepressant treatment, mimicking the observation in humans (Tsankova et al., 2006). In mice, chronic social defeat decreases the expression of two splice variants of *Bdnf* in the hippocampus and induces H3K27 dimethylation, a mark for transcriptional repression (Kouzarides, 2007), in their respective promoter regions (Tsankova et al., 2006). While behavioural anomalies induced by social defeat are reversed by chronic antidepressant treatment, the increase in H3K27 dimethylation is not (Tsankova et al., 2006).

Instead, H3 acetylation and H3K4 methylation, marks of transcriptional activation (Kouzarides, 2007), are increased at the same promoters leading to a reversal of *Bdnf* downregulation (Tsankova et al., 2006; Wilkinson et al.,

2009). Long-term antidepressant treatment also down-regulates the expression of HDAC5 in animals exposed to chronic stress (Renthal et al., 2007). Thus, changes in histone acetylation and methylation by chronic stress at the *Bdnf* gene in the hippocampus are likely important for the development of depressive behaviours, and histone acetylation is a target of antidepressant treatments. Further, chronic unpredictable mild stress (CUMS) also decreases H3K9 methylation at the promoter of corticotrophin-releasing hormone receptor 1 in the rat hippocampus, and is associated with altered hypothalamic-pituitary-adrenal axis, a characteristic of depression (Wan et al., 2014). Further to the hippocampus, other neuroanatomical regions have changes in histone PTMs at genes involved in behavioural response to chronic stress. In ventral striatum, histone PTMs at glial-derived neurotrophic factor gene have been associated with the susceptibility and adaptation to chronic stress in mice (Uchida et al., 2011).

Alterations in DNA methylation have been reported upon antidepressant administration in preclinical and clinical studies. In a rat model of depression, three-week treatment with the selective serotonin re-uptake inhibitor (SSRI) escitalopram reduced mRNA levels of *DNMT1* and *DNMT3a* in forebrain neurons associated with decreased global DNA methylation and partial reversal of pro-depressant behaviors (Melas et al., 2012). Consistently, hypomethylation of the serotonin transporter gene, a main target of SSRIs, affects SSRI treatment response in humans (Domschke et al., 2014). Intraperitoneatal administration of the SSRI fluoxetine leads to increased MeCP2 and methyl CpG-binding domain protein 1 (MBD1) in the adult rat brain (Cassel et al., 2006). Typically, both methyl-binding proteins are enriched in neurons, as compared to glial cells (Zachariah et al., 2012; Zhao et al., 2003). Upon fluoxetine administration, both proteins were found to accumulate in neurons, including GABA-ergic interneurons. This is of particular interest because abnormal GABA-ergic transmission and anomalies in GABA-related gene methylation are linked to major depression and suicide. Depressed patients who committed suicide have higher level of methylation in

the GABA-A α1 receptor subunit promoter, and increased DNMT3b mRNA and protein in the prefrontal cortex when compared to control individuals who died of other causes (Poulter et al., 2008). This suggests the interesting possibility that antidepressant treatments can target the epigenetic machinery in cell types affected by depression. Several studies further reported differential DNA methylation at candidate genes such as *Bdnf* in different tissues of depressed individuals. Increased 5-mC level at *Bdnf* promoter IV is associated with decreased *Bdnf* transcript levels in Wernicke's area of suicide victims, some of whom suffering from MDD (Keller et al., 2010). This suggests that changes in DNA methylation levels could be responsible for *Bdnf* down-regulation in depression. *Bdnf* promoter hypermethylation is observed in both buccal tissue and blood of depressed patients, and is correlated with suicidal behaviour. Treatment of peripheral blood cells isolated from depressed patients with the antidepressant paroxetine reduces DNMT1 activity and *Bdnf* methylation, and correlates with increased *Bdnf* expression (Gassen et al., 2015). *Bdnf* methylation may therefore serve as a biomarker for the diagnostic of MDD (Fuchikami et al., 2011; Januar et al., 2015; Kang et al., 2013). Genome-wide profiling of DNA methylation may also be useful since profiling in blood of patients suffering from depression is distinct from non-depressed individuals (Uddin et al., 2011). But DNA methylation profiling can be variable as revealed by analyses in monozygotic twins from depression-discordant pairs (Byrne et al., 2013; Cordova-Palomera et al., 2015; Davies et al., 2014; Dempster et al., 2014), suggesting the possibility that the methylome of patients with MDD may be more sensitive to environmental influence. Further, miRNAs have also been associated with the long-term effects of chronic stress, an important precursor of depression. Chronic mild stress alters the expression of miR-186 and miR-709 in the hippocampus and pre-frontal cortex in mice (Babenko et al., 2012). miR-19b may mediate the pro-depressive effects of chronic stress by decreasing the expression of adrenergic receptor b1 in BLA (Volk et al., 2014). A possible mode of action of miRNAs is to regulate the molecular pathways underlying stress resilience. MiR-135 is considered an endogenous "anti-depressant" which has been

shown to prevent the development of pro-depressive phenotype in mice by modifying serotonergic activity (Issler et al., 2014). Further, miRNAs can contribute to the regulation of neurogenesis (miR-124 through targeting Sox9; miR-128 through targeting LRRC4C), neurotrophic signalling (miR-26 a and b through targeting *BDNF*) and inflammation (miR-155 and miR-181), which have all been implicated in depression (Dwivedi, 2014; Hutchison et al., 2013; Woodbury et al., 2015). Not surprisingly, clinically efficient anti-depressants lead to notable changes in brain miRNA profiles. For example, fluoxetine promotes the biogenesis of miR-16 in raphe nuclei (Baudry et al., 2010), and imipramine and citalopram regulate miR-1202 (Lopez et al., 2014).

In conclusion, the contribution of epigenetic mechanisms to molecular pathways affected by stress, their role in determining the susceptibility to depression, and their alteration by anti-depressants, highlight their potential role in depressive prophylaxis and therapeutics. It is also possible that non-pharmacological strategies like cognitive behavioural therapy and psychotherapy used to treat depression also implicate epigenetic changes. A recent study showed that response to psychotherapy in patients with borderline personality disorder decreased CpG methylation at two exons of BDNF, BDNF CpG exon I and IV in peripheral blood leukocytes, whereas methylation was increased in non-responders (Perroud et al., 2013). This supports the idea that epigenetic marks are amenable to non-pharmacological treatment approaches in psychiatry, and could serve as therapeutic targets, and prognostic markers in the future. Off note again here is the potential of dietary factors and exercise in prevention and/or treatment of psychiatric disorders. Changes in serotonergic signalling by Tryptophan-rich diets, exercise-induced changes in *BDNF* and induction of neurogenesis are a few ways through which diet and exercise could alter the development or clinical course of depression (Dauncey, 2013, 2014).

2.2.5 Epigenetic dysregulation by environmental stress - the example of early life stress

It has long been recognized that detrimental experiences in early postnatal life have long-lasting consequences for brain functions, especially in the affective domains. The quality of the social and parental environment early postnatal in particular, is a critical determinant of an organism's neuropsychological development. In humans, childhood trauma or prolonged separation from the mother results in deviant behaviors such as drug abuse, in adult life (Khoury et al., 2010). In mice, unpredictable maternal separation combined with unpredictable maternal stress was also shown to lead to severe and persistent emotional and cognitive dysfunctions (Franklin et al., 2010). Such adverse experiences in early postnatal life are known to induce multiple epigenetic modifications in the brain, resulting in altered gene expression (reviewed in (Kundakovic and Champagne, 2015)). Different molecular factors including glucocorticoids and glucocorticoid receptor (GR), mineralocorticoid and mineralocorticoid receptor (MR), arginine vasopressin (AVP) and oxytocin are noteworthy are known to be altered by early traumatic stress.

Maternal care is indeed an important environmental factor for behavioral responses later in life. The offspring of high-nurturing female rat have decreased DNA methylation and increased H3K9 acetylation at *NR3C1*, a gene coding for GR, in the brain (Weaver et al., 2004). In contrast, the offspring of low nurturing females have decreased brain *NR3C1* methylation, suggesting that differential epigenetic marking underlies changes in GR expression by maternal care. In humans, childhood maltreatment and adversity also lead to alterations of DNA methylation at *NR3C1* as observed in the hippocampal autopsy specimens of suicide victims with a history of childhood abuse (McGowan et al., 2009). Importantly, methylation profile at *NR3C1* promoter in response to early life trauma is comparable in rats and humans (Suderman et al., 2012). It is not surprising that epigenetic regulation of GR Is under stringent regulation considering its critical role in mediating the effect of stress on the brain, and serving as an important determinant of

neuro-endocrine integration (Woodbury et al., 2015). Likewise, disrupted maternal care in early childhood decreases hippocampal MR expression in adults in association with changes in histone PTMs (Gapp et al., 2014). Parental contact in early postnatal life also determines DNA methylation of oxytocin gene in the brain and the periphery of the offspring in rodents (Hammock, 2015). Finally, periodic mother-infant separation in mice leads to DNA hypomethylation in the promoter region of AVP, which correlates with an increased and persistent expression of AVP resulting in increased vulnerability to stress in adulthood (Hammock, 2015; Kember et al., 2012). Such persistent increase in avp could have pleiotropic effects on adult brain functions related to its role in regulation of social behaviors, pair bonding, sexual preferences, and its noted dysregulation in anxiety and depression (Meyer-Lindenberg et al., 2011).

Genome-wide CpG promoter methylation screen revealed differentially methylated regions in post-mortem hippocampal tissue of suicide victims with a history of childhood abuse. These changes are associated with the dysregulation of a network of genes involved in neuronal plasticity (Labonté et al., 2012). In adult monkey, early-life maternal deprivation is associated with changes in DNA hydroxymethylation at the promoter of genes related to psychiatric disorders and/or perinatal adversity in cortex (Massart et al., 2014). In maternally deprived animals, hydroxymethylation was decreased at the promoter of the dopaminergic receptor 3, the adrenoreceptor alpha 1, and the serotonergic transporter. In contrast, hydroxymethylation was increased at the promoter of the GABAA receptor alpha 2, the transcriptional repressor REST and the MAP kinase 1. Hydroxymethylation within promoter regions has been hypothesized to constitute an epigenetic mark presumably associated with transcriptional modulators (Cartron et al., 2013; Mellen et al., 2012). It should be noted however, that further studies are required to fully understand its functional implications. Altogether, these findings suggest that early life adversity triggers variations in both DNA methylation and hydroxymethylation that persist into adulthood.

Besides DNA methylation, miRNAs may also be important mediators of the effects of early postnatal environmental influence, since several of them are affected by early adversity. miR-16 expression is increased in the rat hippocampus after early life trauma, leading to decreased hippocampal BDNF (Bai et al., 2012). Maternal deprivation in early childhood also alters miR-504 expression in nucleus accumbens in rats (Zhang et al., 2013c).

Epigenetic modifications can carry the effects of early life trauma not only through life but also across generations. Exposure to early life trauma induced by unpredictable maternal separation and unpredictable maternal stress has been shown to alter behavior and metabolism across generations, even in the absence of any traumatic exposure in the offspring. Transmission of these effects implicates sperm RNAs since sperm RNAs from traumatized males injected into naïve control fertilized oocytes recapitulate the effects of trauma in the resulting animals. These RNAs include miRNAs, which expression is altered by trauma not only in sperm, but also in the serum and brain of exposed mice, and in the brain of the offspring (Gapp et al., 2014). Apart from miRNAs, early life trauma also alters DNA methylation in sperm and brain across generations. It alters DNA methylation in several genes, such as corticotropin-releatsing factor receptor 2 and cannabinoid receptor 1, associated with depressive-like behaviors, in the brain and the sperm of exposed males and their offspring (Franklin et al., 2010), suggesting another potential means of transgenerational epigenetic inheritance. Such epigenetic alterations were recently shown to be reversed by environmental or pharmacological manipulations. Cross-fostering of pups, or treatment with the HDAC inhibitor trichostatin A leads to *NR3C1* DNA hypomethylation and histone hyperacetylation in the offspring of low nurturing mothers (Weaver et al., 2004). Likewise, methyl supplementation via administration of L-methionine, a SAM precursor, can reverse maternal programming of stress responses via GR (Weaver et al., 2005). Importantly, sub-optimal maternal care also decreases histone acetylation of a large number of genes in the

mouse hippocampus, including *ATRX* and *Reelin*, and these changes are reversed by treatment with trichostatin A. (Weaver et al., 2006). Finally, paternal environmental enrichment also reverses the transmission of behavioral traits, and corrects aberrant DNA methylation of *NR3C1* in a mouse model of early life trauma (Gapp et al., 2016).

Future research on this immensely intriguing topic should focus on the effectors of these epigenetic changes, and on identifying the mechanisms underlying the effects of early life-trauma on the brain and the germ-line. Circulating hormones and cytokines could be relevant as they can readily access the brain and germ cells. Indeed, maternal immune activation mediates the transgenerational effects of prenatal stress (Weber-Stadlbauer et al., 2016). Further, the observation that environmental enrichment can reverse some of the long-term adverse consequences of early postnatal trauma through epigenetic modulation also raises the question if dietary factors could have similar preventive or therapeutic benefits. Influence of folate-containing diet to modulate DNA methylation, tryptophan-rich diet to modify serotonergic signaling in the brain, and anti-oxidant and anti-inflammatory properties of certain foods are important to consider (Dauncey, 2013, 2014, 2015). Although the field is extremely dynamic and the subject of considerable research, more pre-clinical and clinical research will be needed for applying these findings to the clinic.

2.3 Conclusions and outlook

It is becoming increasingly clear that epigenetic mechanisms play a pivotal role in higher-order brain functions, in both physiological and pathological conditions. Epigenetic mechanisms contribute to the etiopathology of brain disorders, determine an individual's disease susceptibility, and underlie the therapeutic efficacy of treatments. In some cases, epigenetic mechanisms serve as vectors carrying the effects of environmental stressors to subsequent generations. A precise delineation of these processes is necessary for a

better understanding of the mechanisms underlying brain diseases, and for the development of potential treatments and preventive strategies. Future research should therefore explore how epigenetic mechanisms contribute to the risk to or protection against brain disorders. Possible cross-talks between epigenetic mechanisms such as regulation of HDACs or DNMTs binding by ncRNAs, or potential master epigenetic regulators and functional 'epigenetic codes' should be identified. Further to DNA methylation, histone PTMs, and miRNAs, other forms of epigenetic regulation such as long non-coding RNAs, piwi RNAs (piRNAs), RNA methylation or prion-like mechanisms may also be involved. Long non-coding RNAs were found to be altered in mouse models of AD (Lee et al., 2015) and depression (Huang et al., 2016). Similarly, piRNAs have emerged as critical vectors of inter-generational inheritance in *C. elegans* and Drosophila, and as regulators of long-term memory in mice (Ashe et al., 2012; Rajasethupathy et al., 2012). These processes merit attention in the future.

It is also important to consider and when possible solve the issue of the use of epigenetic treatments for brain disorders. The complexity of the epigenetic landscape in the brain, and of its regulation in different brain cell types during development and adulthood, with constant interaction with the environment needs to be carefully studied (Szyf, 2015). Additional challenges include the possibility that manipulating an epigenetic effector could have contrasting effects in different brain regions and/or functions, or in the periphery (Szyf, 2015). A relevant example is that of the miRNA cluster miR183-96-182, which promotes memory formation when over-expressed in the mouse hippocampus (Woldemichael et al., 2016), but has also been linked with depressive-like behavior in rat (Li et al. Prog Neuropsychopahrm Biol Psych 2016). An additional challenge is the low permeability of the blood-brain barrier. Some of the challenges of using epigenetic drugs to treat brain diseases need to be addressed in the future, and consider the mode of regulation of epigenetic enzymes and their targets in different brain cells, neuroanatomical regions, and functional contexts. It would also be important to determine if epigenetic

therapies are more likely to benefit selected groups based on genetic features like the presence or absence of metabolizing enzymes, in which case those groups would need to be carefully isolated through genetic screening and/or clinical histories.

In summary, the importance of epigenetic dysfunction in brain diseases is now fully appreciated. With a better understanding of their modes of regulation and the identification of their specific downstream targets, and their region and cell-type specific effects, safe and efficient epigenetic treatments can be envisaged for treatment of brain disorders in future.

2.4 Acknowledgments

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3 Novel interplay between TETs and microRNAs in the adult brain for memory formation.

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3.1 Abstract

5-hydroxymethylation (5-hmC) is an epigenetic modification on DNA that results from the conversion of 5-methylcytosine by Ten-Eleven Translocation (TET) proteins. 5-hmC is widely present in the brain and is subjected to dynamic regulation during development and upon neuronal activity. It was recently shown to be involved in memory processes but currently, little is known about how it is controlled in the brain during memory formation. Here, we show that *Tet3* is selectively up-regulated by activity in hippocampal neurons *in vitro*, and after formation of fear memory in the hippocampus. This is accompanied by a decrease in miR-29b expression that, through complementary sequences, regulates the level of *Tet3* by preferential binding to its 3'UTR. We newly reveal that SAM68, a nuclear RNA-binding protein that regulates splicing, acts upstream of miR-29 by modulating its biogenesis. Together, these findings identify novel players in the adult brain necessary for the regulation of 5-hmC during memory formation.

3.2 Author summary

DNA hydroxymethylation is mediated by the family of Ten-Eleven Translocation (TET) proteins comprising TET1, 2 and 3, and is associated with transcriptional gene regulation. Recent studies have suggested a role for TETs in the epigenetic regulation of gene expression during memory formation. However, the mechanisms of such regulation remain unknown. MicroRNAs are short non-coding RNAs that regulate gene expression transcriptionally and/or translationally, and provide a rapid and reversible mode of control. Here, we show that both, *Tet3* and the miR-29 cluster are differentially regulated, in an opposite manner, in the hippocampus after learning, and in cultured hippocampal neurons upon activity. MiR-29b, one member of the cluster, binds to the 3'UTR of *Tet*s and controls their mRNA level, but has a preference for *Tet3*. We identify a novel regulator of the biogenesis of miR-29 cluster, the RNA-binding protein SAM68, and show that it controls the transcription of the cluster. These findings reveal a new cascade involving SAM68, miR-29 cluster and TET3 in the epigenetic regulation of memory genes after learning.

3.3 Introduction

Epigenetic mechanisms involving DNA methylation are essential for the regulation of gene expression in the brain, and are required for learning and memory formation (Levenson and Sweatt, 2005). Until recently, DNA methylation was believed to be stable in post mitotic cells, but it is now known to be dynamically regulated at specific sites upon neuronal stimulation and learning (Guo et al., 2011b; Ma et al., 2009; Miller and Sweatt, 2007), indicating that it is reversible. While DNA methyltransferases (DNMTs) catalyze DNA methylation on position 5 of cytosines (5-methylcytosine or 5mC), ten-eleven translocation methylcytosine dioxygenases (TETs) are

responsible for DNA demethylation. TET proteins (TET1, 2 and 3) allow demethylation by converting 5mC into 5-hydroxymethylcytosine (5hmC). TETs can further oxidize 5hmC into 5-formylcytosine and 5-carboxylcytosine that is subsequently excised by the base excision repair pathway (Ito et al., 2011). 5hmC accumulates in the brain during development and is present at high level in the adult brain, suggesting that it likely plays an important role (Szulwach et al., 2011). Like DNA methylation, it is dynamically regulated by neuronal activity (Guo et al., 2011c) but the mechanisms that allow its dynamic regulation are not known.

TET1 is the best-characterized enzyme among the TET family with regard to learning and memory. *Tet1* mRNA was shown to be downregulated 1 and 3 hours after contextual and cued fear conditioning in area CA1 of the dorsal hippocampus (Kaas et al., 2013). TET1 regulates the expression of several activity-dependent genes implicated in learning and memory and its overexpression in the hippocampus impairs long-term associative memory [8]. However, global TET1 knockout in mice does not alter memory acquisition and consolidation, but selectively impairs the extinction of hippocampus-sensitive memories (Rudenko et al., 2013). It also affects neurogenesis (Zhang et al., 2013b) and long-term depression in the hippocampus (Rudenko et al., 2013).

Less is known about the role of TET3 in memory processes. However, in the cortex and hippocampus, two brain regions essential for learning and memory, *Tet3* is the most highly expressed enzyme of the TET family enzymes (Szwagierczak et al., 2010). *Tet3* but not *Tet1* mRNA increases 2 hours after extinction training in prefrontal cortex, a structure critical for memory extinction (Li et al., 2014b). *Tet3* mRNA increases 2 hours after extinction training in the prefrontal cortex, and knockdown of *Tet3* in this region impairs memory extinction, without affecting learning (Li et al., 2014b). Thus, TETs might have various roles in memory processes depending on the brain region, and possibly the type of memory.

The modes of regulation of TETs remain unknown but microRNAs (miRNAs) have been thought as potential candidates. miRNAs are short non-coding RNAs that can control neuronal gene expression required for memory formation. The biogenesis, rapid turnover and combinatorial modes of action of miRNAs make them ideal candidates for a dynamic and reversible regulation of gene expression (Ha and Kim, 2014). They can control multiple targets simultaneously through degradation of their mRNAs or translational repression. Some miRNAs have also been implicated in the regulation of DNA methylation directly by targeting *Dnmts* or indirectly by acting on transcription factors that control *Dnmts* transcription (Benetti et al., 2008)(Fabbri et al., 2007). The miR-29 family (a, b and c), in particular, was shown to contribute to epigenetic regulation in cancer by targeting *Dnmt3a* and *b* (Fabbri et al., 2007). Conversely, miRNAs themselves are subject to specific mechanisms of control. Their transcription, processing and degradation are regulated by different processes involving protein-protein and protein-RNA interactions (Krol et al., 2010b). In these mechanisms, RNA-binding proteins (RBPs) are very important regulators implicated in different stages of miRNAs biogenesis, localization, activity and degradation. SAM68 (also called KHDRBS1), is an RBP mostly known to regulate activity-dependent alternative splicing (Iijima et al., 2011), was recently shown to influence the expression of a subset of miRNAs in male germ cells (Messina et al., 2012). In this study, we provide evidence that the miR-29 family is differentially regulated in the adult hippocampus upon learning and that miR-29 biogenesis is modulated by SAM68. These miRNAs are involved in the control of *Tets*, in particular *Tet3*, which itself is regulated in an activity-dependent manner upon learning to induce 5hmC.

3.4 Results

To determine the dynamics of TETs regulation upon neuronal activity in the adult brain, we quantified the level of *Tet* mRNAs in the hippocampus after contextual fear conditioning (CFC) (S1 Fig). While *Tet1* and *Tet2* mRNA remained unchanged after conditioning, *Tet3* mRNA was up-regulated after 30 min and 3 h but returned to baseline after 24 h (Fig 1a). To test whether the changes in *Tet3* expression were specific to memory formation in CFC and were not related to the stress response elicited by fear conditioning, we examined the effects of acute cold swim stress on *Tet*s expression in the hippocampus. *C-fos*, an activity-dependent immediate early gene, was used as positive control to ensure that neuronal activation occurred (S2a Fig). Importantly, *Tet3* expression was not modified by cold swim stress (S2b Fig). Further, *Tet3* expression was also up-regulated by activity in cultured hippocampal neurons *in vitro*. Neuronal activation by NMDA, confirmed by *C-fos* expression (S3a Fig), significantly increased *Tet3* expression after 5 min and 1 h (Fig 1b). It also slightly increased *Tet2* expression after 1 h but decreased *Tet1* after 5 min, suggesting a dissociated response of the three TETs. Consistently, *C-fos, Tet2* and *Tet3* but not *Tet1* were also up-regulated by activation of NMDA receptors by the co-agonist glycine *in vitro* (S3b, c Fig). These findings suggest that NMDA receptor signaling increases *Tet3* transcription, both after neuronal activation *in vitro* and learning *in vivo*.

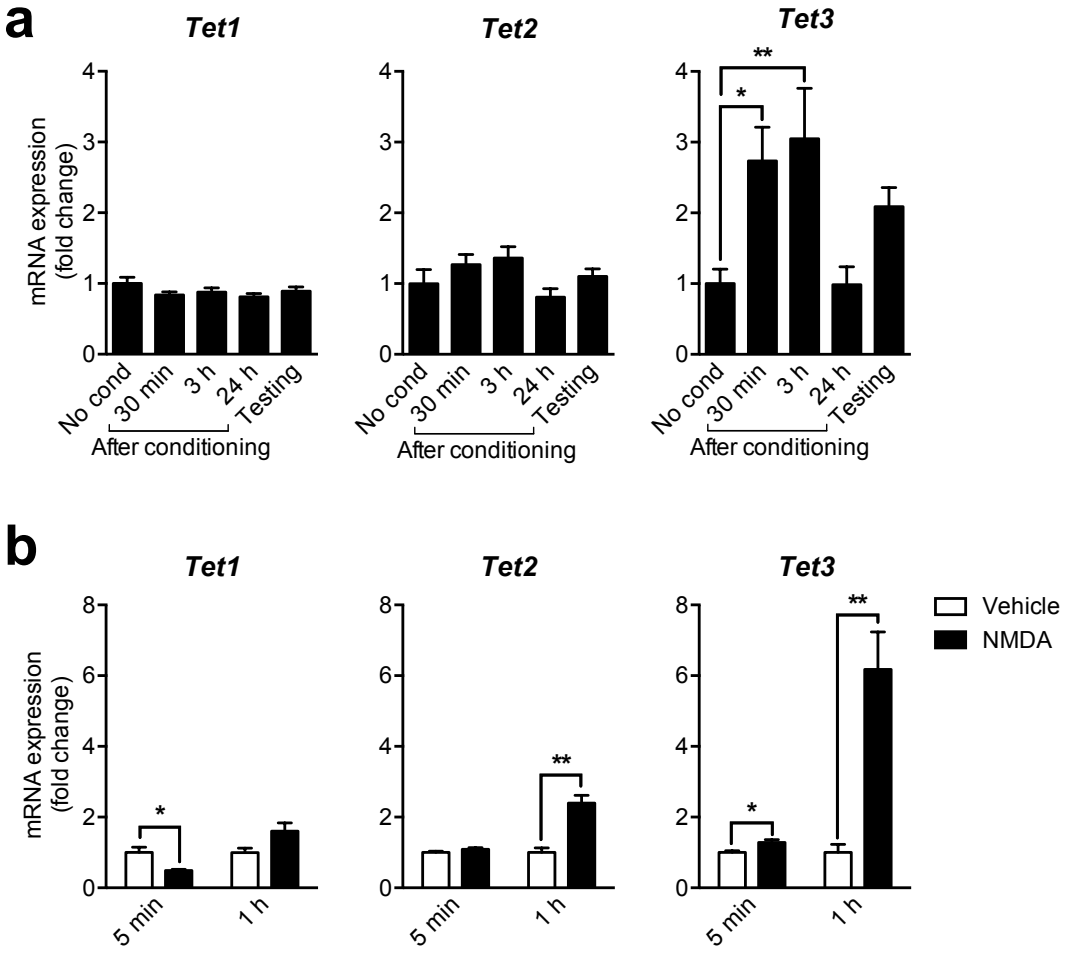

Figure 3-1 Activity-dependent expression of *Tet* genes. (**a**) Level of *Tet1*, *2*, and *3* in the hippocampus 30 min, 3 h, 24 h after fear conditioning, or 30 min after testing 24 h following conditioning, measured by real-time quantitative reverse transcription-PCR (RT-qPCR). (**b**) Level of *Tet1*, *Tet2* and *Tet3* in hippocampal primary neurons 5 min and 1 h after NMDA stimulation (60 µM, 5 min) measured by RT-qPCR. No cond., no conditioning; *p<0.05, **p<0.01. Data represent mean s.e.m. Supporting information can be found in S1 Fig, S2 Fig and S3 Fig.

We next sought to identify which mechanisms participate to the control of *Tet3* mRNA level. I*n silico* target gene prediction algorithms indicated that *Tet* 3'-UTR has multiple well-conserved binding sites for miR-29 (TargetScan analysis, S1 Table). Therefore, we examined whether miR-29, a miR cluster including miR-29a, b and c, has a relation to *Tets* during CFC. While miR-29a and c remained constant, miR-29b expression was significantly down-regulated 3 h after conditioning and following testing (Fig 2a). MiR-29b was

also decreased 5 min and 1 h after NMDA stimulation in hippocampal neurons, while miR-29a and c expression decreased only after 1 h (Fig. 2b). Likewise, it was decreased after 5 min and 1 h of glycine treatment while miR-29a and c were not (S4 Fig). These results suggest that miR-29b expression is activity-dependent and has an expression profile inverse to *Tet3* after learning and neuronal activity.

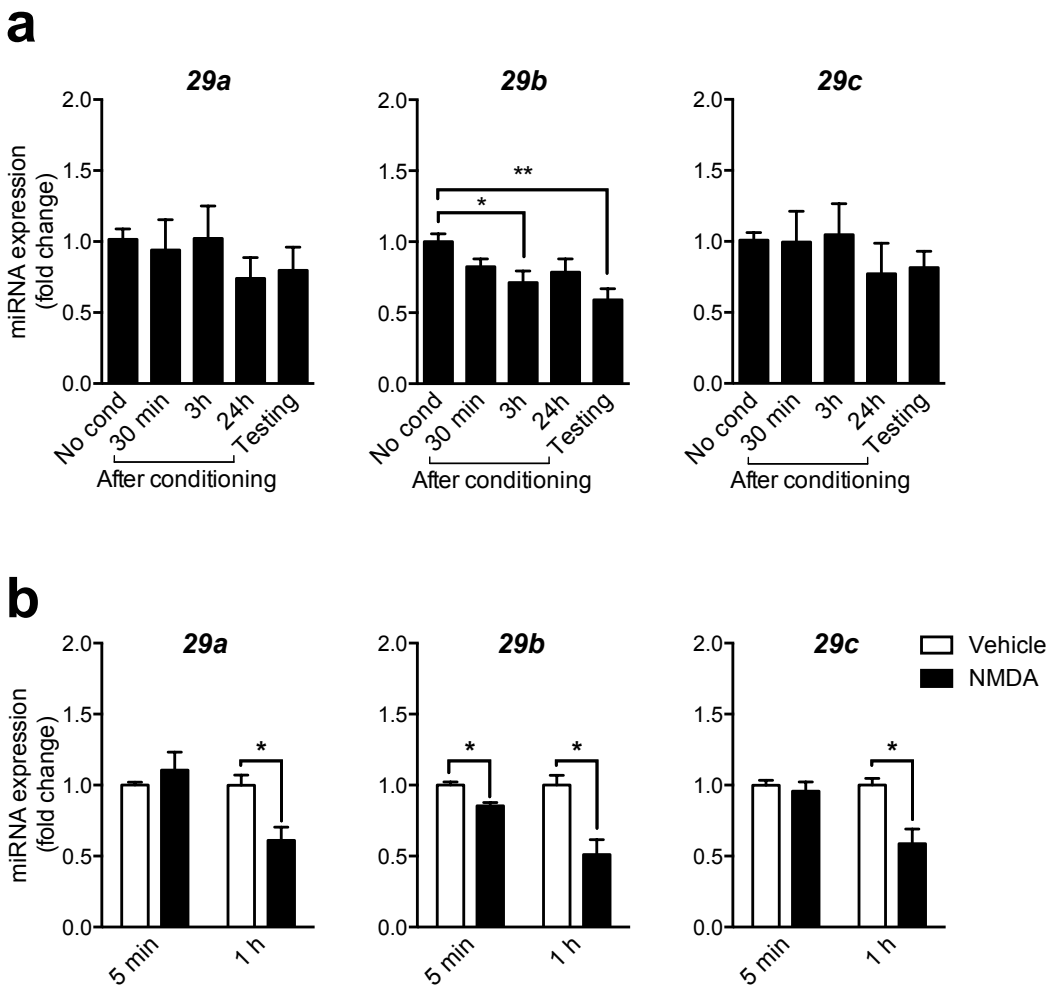

Figure 3-2 Activity-dependent expression of *miR-29* family. (**a**) Level of *miR-29a, b* and *c* in the hippocampus 30 min, 3 h, 24 h after fear conditioning, or 30 min after testing (24h after conditioning) measured by RT-qPCR. (**b**) Level of *miR-29a*, *b* and *c* in hippocampal primary neurons 5 min and 1h after NMDA stimulation (60 µM, 5 min) measured by RT-qPCR. No cond., no conditioning; **p*<0.05, ***p*<0.01. Data represent mean s.e.m. Supporting information can be found in S4 Fig.

To test if miR-29b targets *Tets*, we manipulated its level in N2a cells using miRNA mimic or antagomir. Overexpression of a miR-29b mimic down-regulated *Tet1, 2* and *3* expression (Fig. 3a) while miR-29b knockdown increased *Tet1* and *3* expression (Fig. 3b). Then, using two different regions of *Tet3* 3'-UTR (*Tet3*(1) and *Tet3*(2)) with seed sequences for miR-29 cluster (S5a Fig) and a luciferase reporter, we further examined the interaction between *Tet3* and miR-29b. MiR-29b mimic significantly reduced luciferase activity with both reporters (Fig. 3c), showing that miR-29b regulates the level of *Tet3* mRNA likely by binding to its 3'UTR. Additionally, high doses of miR-29b mimic reduced the luciferase activity of *Tet1* and *Tet2* reporters (S5b Fig).

To determine whether miR29b preferentially regulates *Tet3*, we tested the effect of graded concentrations of miR-29b mimic. While overexpression of miR-29b by mimic had no effect on the control vector (S6 Fig), it decreased the expression of *Tet3* luciferase reporters in a dose-dependent manner. At low concentration (0.1 nM), the miR-29b mimic repressed *Tet3* luciferase reporters but had no effect on *Tet1* and *Tet2*, indicating a dose-dependent target regulation of miR-29b (Fig. 3d). Using STarMiR [19] to model mRNA secondary structure, we found that *Tet3* 3'UTR contains more accessible miR-29b binding sites than *Tet1* and *Tet2* 3'UTRs (S2 Table), providing a potential explanation for our experimental findings. As expected, miR-29b overexpression decreased *Dnmt3a* and *b* mRNA level as well as the luciferase activity of a *Dnmt3a* reporter (S7a, b Fig). However, *Dnmt1* was not changed, consistent with the absence of binding sites for miR-29s in its 3'-UTR (S7a Fig), confirming the specificity of the assay.

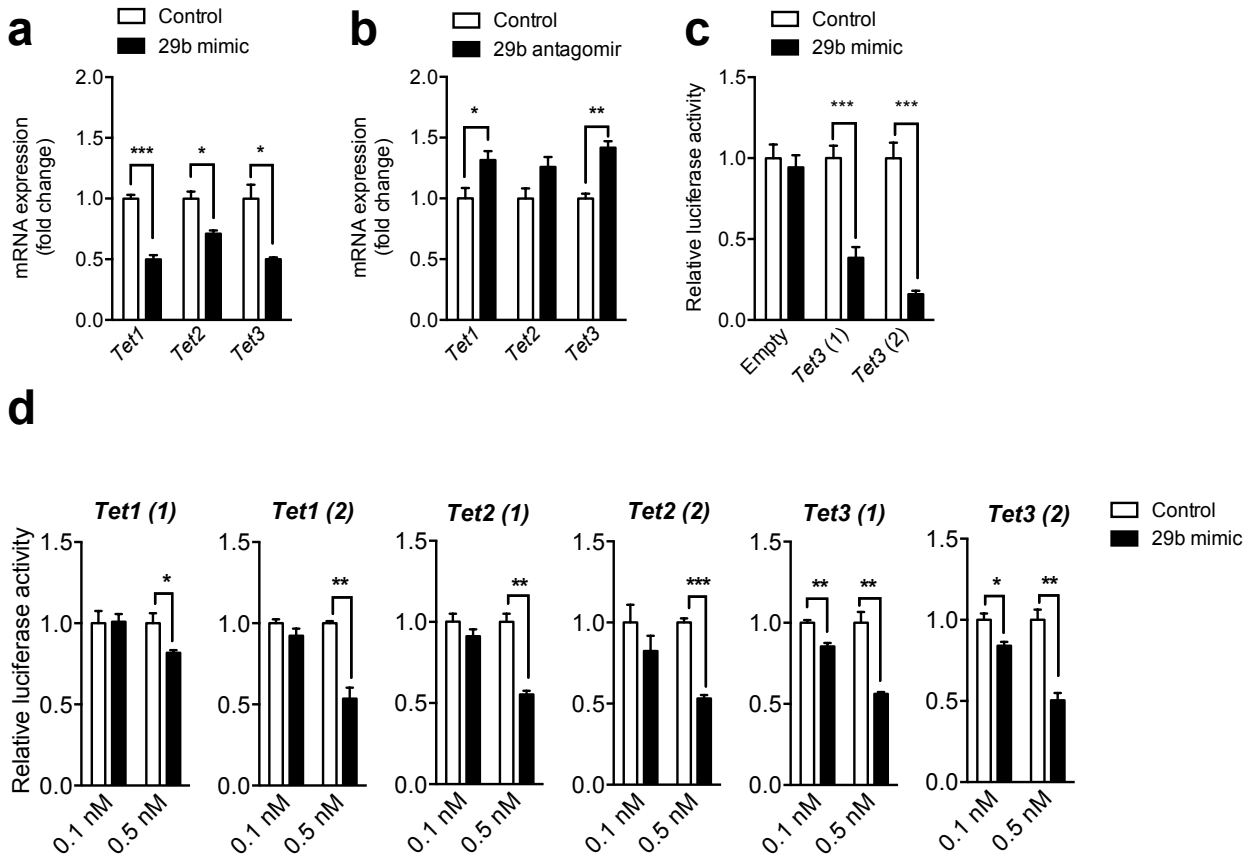

Figure 3-3 MiR-29b preferentially controls *Tet3* expression level. (**a**) Level of *Tet1*, *Tet2,* and *Tet3* in N2a cells after transfection with miR-29b mimic or control measured by RT-qPCR. (**b**) Level of *Tet1*, *Tet2,* and *Tet3* in N2a cells after transfection with miR-29b antagomir or control measured by RT-qPCR. (**c**) Analysis of *Tet3* luciferase reporters in the presence of miR-29b mimic or control (60 nM) in N2a cells (**d**) Analysis of *Tet1, Tet2 and Tet3* luciferase reporters in the presence of graded concentrations of miR-29b mimic or control in N2a cells. **p*<0.05, **p<0.01, ****p*<0.001. Data represent mean s.e.m. Supporting information can be found in S5 Fig, S6 Fig and S7 Fig.

To further investigate the function of TET3, we examined the effects of *Tet3* knockdown on the expression of potential candidate genes. Using siRNAs specifically targeting *Tet3* (*Tet1* and *2* remained unchanged, S8 Fig), we identified genes involved in Notch signaling (*Notch1* and *2*), repair-based DNA demethylation (*Gadd45a* and *b)* and transcriptional activation (*Elk1, Crebbp* and *Creb1*) (Fig.4). Interestingly, all these genes were previously implicated in neuronal activity, synaptic plasticity and/or memory processes (Ahi et al.,

2004; Dias et al., 2014; Sultan et al., 2012). In contrast, the expression of major components of the miRNA biogenesis *(Drosha*, *Dgcr8*, *Dicer)* and other microprocessor accessory proteins involved in the control of miRNA biogenesis such as *p68*, *p72* and *p53* were not altered by *Tet3* knockdown (Fig. 4), indicating that TET3 does not affect global miRNA biogenesis. These results suggest that an intermediate molecular player is involved in modulating the expression of miR-29b upon neuronal activity.

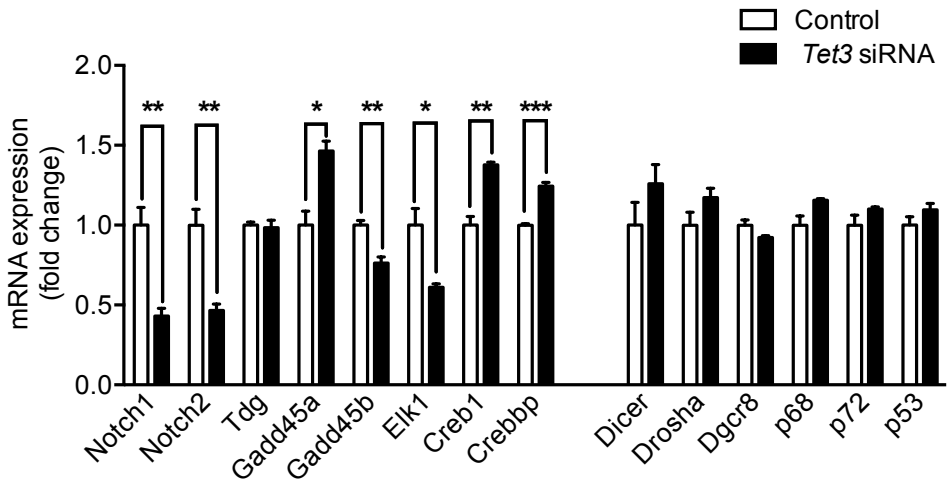

Figure 3-4 TET3 regulates the expression of synaptic and memory-related genes. Transcriptional analysis of genes involved in synaptic plasticity, memory formation, and miRNA biogenesis after *Tet3* knockdown in N2a cells by RT-qPCR. *p<0.05, **p<0.01, ***p<0.001. Data represent mean s.e.m. Supporting information can be found in S8 Fig.

Previous work has identified that SAM68 – an RBP - influenced the expression of specific miRNAs in male germ cells, including miR-29b (Messina et al., 2012). RBPs play a role in the biogenesis of specific miRNAs (reviewed in (Loffreda et al., 2015)), thus we examined the potential link between SAM68 and its role in regulating the biogenesis of the miR-29 family. MiRNAs are produced through the action of multiple enzymatic steps involving the transcription of primary miRNAs (pri-miRNAs), their processing into

precursors miRNAs (pre-miRNAs) in the nucleus and then into mature miRNAs in the cytoplasm (Ha and Kim, 2014).

To determine whether miR-29 biogenesis is modulated by SAM68, we quantified the level of pre- and mature miR-29s after *Sam68* knockdown. We found that pre-miR-29b and pre-miR-29c were significantly up-regulated in N2a cells (Fig. 5a). Similarly, *Sam68* knockdown (S9a Fig) led to an increase in mature miR-29a, b and c, confirming that SAM68 is implicated in the biogenesis of these miRNAs (Fig. 5a). Although previous studies found that miR-182 and miR-10b are dynamically regulated after fear conditioning (Griggs et al., 2013; Kye et al., 2011) the expression of these miRNAs was not affected by *Sam68* knockdown, showing a clear selectivity of the effect (S9b Fig). Since primary, precursor and mature miR-29b were up-regulated upon *Sam68* knockdown, it indicates that SAM68 likely exerts its regulatory effect at the transcriptional level.

To further investigate the mechanisms by which SAM68 modulates miR-29 biogenesis, we used the transcription inhibitor actinomycin D (ActD) to test whether transcription is responsible for the up-regulation of precursors and mature forms of miR-29b. While *Sam68* knockdown led to an increased level of pri-miR-29a/b-1 transcripts, as well as, pre-miR-29b and pre-miR-29c, ActD treatment blocked these changes (Fig. 5b, c). This suggests that SAM68 likely acts upstream of RNA Pol II-dependent transcription to regulate miR-29b. To determine whether the expression of *Sam68* is regulated by activity, we quantified the level of *Sam68* transcripts in hippocampal neurons after NMDA stimulation. *Sam68* was significantly increased 5 min after neuronal activity, but not 1 h later (S10 Fig). Therefore, we propose that transient increase in *Sam68* inhibits pri-miR-29a/b transcription, thereby reducing mature miR-29b levels thus reinforcing *Tet3* transcriptional program.

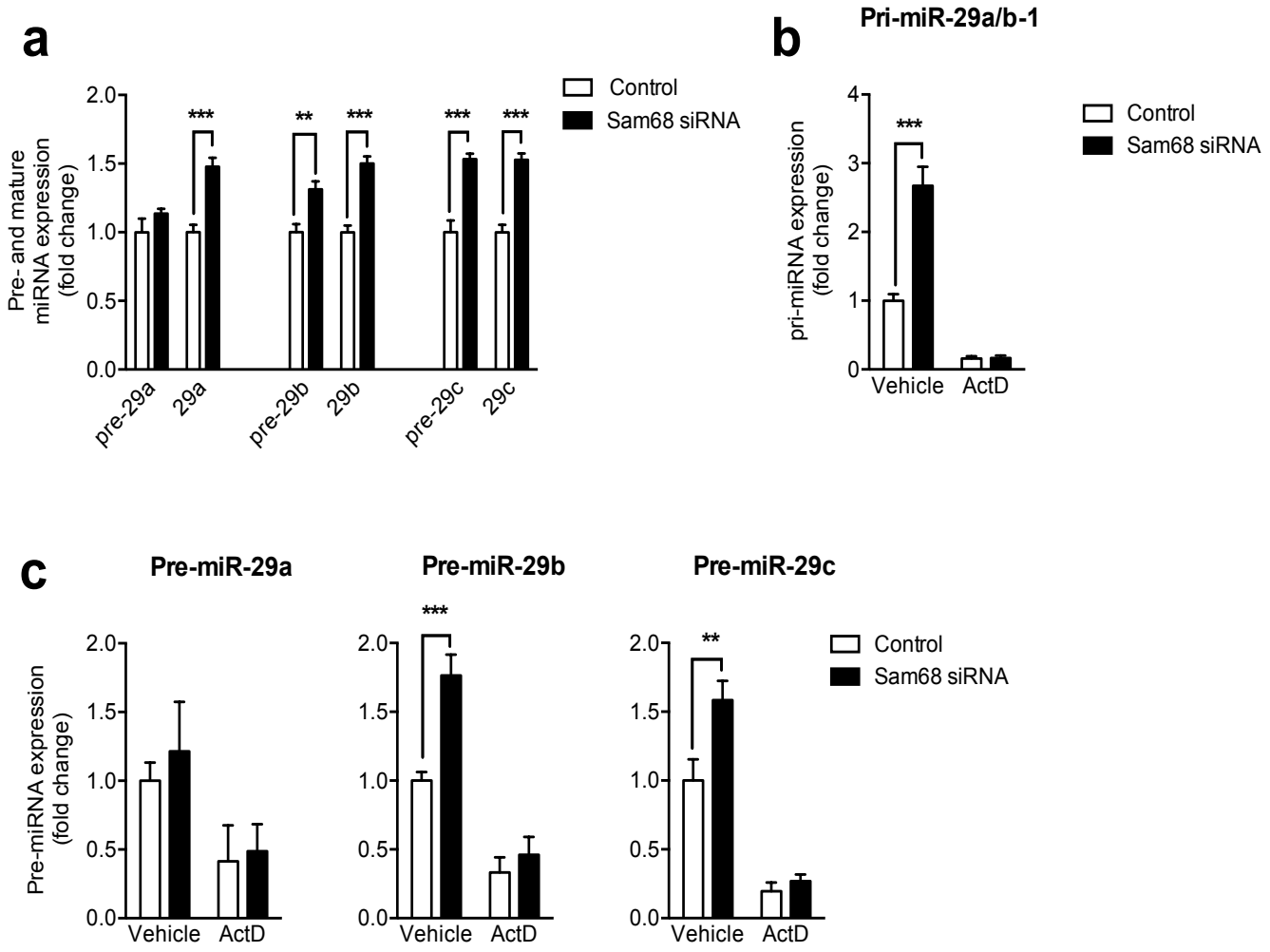

Figure 3-5 SAM68 modulates the biogenesis of miR-29s at the transcriptional level. (**a**) Level of precursor and mature miR-29a, b and c in N2a cells after *Sam68* knockdown. **p<0.01, ***p<0.001 determined by unpaired t test (**b**) Level of nuclear pri-miR29a/b-1 transcripts in N2a cells after *Sam68* knockdown measured by RT-qPCR in the presence of ActD treatment or vehicle. (**c**) Level of pre-miR-29a, b and c in N2a cells after *Sam68* knockdown measured by RT-qPCR in the presence of ActD treatment or vehicle. **p<0.01, ***p<0.001 determined by two-way ANOVA followed by Bonferroni's post-hoc test. Data represent mean ± s.e.m.

3.5 Discussion

The present data demonstrate that *Tet3* expression is preferentially increased in the hippocampus after learning. This effect is, however, transient, as *Tet3* mRNA levels come back to baseline 24 h after training. Neuronal stimulation through activation of NMDA receptors in primary hippocampal neurons increases *Tet3* mRNA levels, indicating NMDA-dependent regulation of *Tet3* expression. These results are consistent with previous data showing regulation of *Tet3* expression upon global synaptic activity changes (Yu et al., 2015). Thus, *Tet3* activity-dependence may explain its up-regulation in the hippocampus after learning. Although *Tet2* expression is not altered in the hippocampus after training, it is significantly up-regulated upon NMDA stimulation in primary neurons, suggesting that *Tet2* is also activity-dependent. It is possible that this up-regulation is neuron-specific, but may also affect glial cells. In agreement with previous reports, *Tet1* transcripts were found to decrease *in vitro* (Kaas et al., 2013). Although Kaas *et al.* confirmed the effect *ex vivo* using the CA1 subregion of the hippocampus, we did not observe any significant change in the whole hippocampus in adult mice. Even though all TETs share the same enzymatic activity, TETs seem to be differentially recruited in specific sub-regions of the hippocampus upon learning, and thus, may contribute to different biological processes. Following acute stress, the level of *Tet3* transcripts in the hippocampus was not changed, indicating that *Tet3* is specifically regulated upon learning but not stress.

The change in *Tet3* expression level after CFC inversely correlates with that of its targeting miRNA, miR-29b. Stimulation of NMDA receptors in cultured hippocampal neurons also lead to alterations in miR-29 expression levels, indicating that changes in miR-29s, similarly to *Tets*, occur in an NMDA dependent-manner. Changes in miR-29b expression likely orchestrate a temporal regulation of *Tet* expression associated with learning by either releasing its mRNAs from miRNA-mediated destabilization, and/or acting as a

fine-tuner of gene expression by reinforcing *Tet3* transcriptional program. Several miRNAs have been previously reported to exhibit dynamic expression following neuronal activity (Kye et al., 2011), suggesting that coordinated changes in miRNA expression contribute to the regulation of newly synthetized activity-dependent mRNA targets. Specifically, neuronal activity was found to decrease the expression of most neuronal miRNAs in the hippocampus (Eacker et al., 2011). This is in line with a regulatory network in which miRNAs maintain transcripts in a repressed state until relieved by neuronal activity.

Threshold response in target gene expression by miRNAs has been proposed as a mode of gene regulation by miRNAs (Mukherji et al., 2011). Therefore, if the pool of *Tet1, 2* and *3* mRNA is below the saturation regime of miR-29b, then all *Tets* will be similarly repressed regardless of expression level. However, as *Tet3* mRNA level raises following neuronal activity, target de-repression due to miR-29b saturation might occur. As *Tet1* mRNA level drops upon neuronal activity, *Tet1* is likely to be subjected to constant repression. Interestingly, a study reported that gene transcripts up-regulated after CFC have more predicted miRNA binding sites in their 3'-UTR than down-regulated ones (Kye et al., 2011), suggesting that down-regulated transcripts are less likely to be regulated by miRNAs. In agreement with this observation, *Tet1* 3'-UTR has fewer putative miR-29 binding sites than *Tet3* 3'-UTR even if longer (S1 Table).

Although miR-29b regulates mRNA levels of all members of the TET family through complementary binding to their 3'UTRs, we found that a low amount of miR-29b preferentially regulates *Tet3* and to a lesser extent *Tet2* while a higher amount affects all *Tets*. The extent of target gene repression by miRNAs depends on the expression level of individual miRNAs, as well as, their targets. In support of this idea, TET1 has low expression in the adult brain, while TET2 and 3 are relatively abundant, in particular in the hippocampus and cortex (Szwagierczak et al., 2010). Additionally, miR-29a

and b have a relatively broad expression pattern in the brain (Hebert et al., 2008; Ouyang et al., 2013). The number, position, and co-operation of miRNA binding sites within the 3'UTR, and the secondary structures of target mRNAs are additional relevant factors that determine the strength of miRNA-mediated gene repression (Kertesz et al., 2007; Krek et al., 2005; Long et al., 2007; Saetrom et al., 2007). Accordingly, the predicted secondary mRNA structure of *Tet3* contains more accessible binding sites for miR-29b than *Tet1* and *Tet2* (S2 Table), suggesting that *Tet3* is more likely to be under the regulation of miR-29b. Consistently, *Tet3* mRNA in the hippocampus was found to be highly enriched in the fraction of AGO2-bound mRNAs while *Tet1* could not be detected, suggesting that in the brain *Tet3* is more likely to be a target of miRNAs (Malmevik et al., 2015).

This study demonstrates that miR-29b is important for the regulation of *Tet* expression and miR-29b itself is subjected to regulation, as its level decreases following learning. The biogenesis of miRNAs is extensively controlled by protein interactors to ensure cell/tissue specific functions or appropriate response to stimuli. One of the largest groups of proteins that has been recognized as important modulator of miRNA biogenesis and function are RBPs. We provided evidence that the RBP SAM68 is involved in the biogenesis of the miR-29 family. As *Sam68* knockdown leads to increased expression of pri-miR-29a/b, precursor and mature miR-29b, SAM68 is likely to mediate its negative regulatory effect at the transcriptional level. Consistently, SAM68 was previously shown to play a role in transcriptional regulation that is independent of its RNA binding activity (Hong et al., 2002; Li and Richard, 2016). We further found that *Sam68* expression is transiently regulated by neuronal activity in hippocampal neurons. Previous work has shown that neuronal activity triggers activation of SAM68 through phosphorylation at serine 20 (Iijima et al., 2011), providing evidence that SAM68 is controlled in an activity-dependent manner. Consequently, SAM68 is likely to modulate miR-29b transcription upon neuronal activity, ultimately

leading to reduced mature miR-29 levels, thus allowing *Tet3* transcripts to increase.

Activity-dependent increase of *Tet3* in the hippocampus after CFC is proposed to impact on the transcriptional activity of genes related to contextual memory formation. In line with this hypothesis, TET3 was recently identified as a critical regulator of activity-induced gene expression in cultured neurons. In this study, a high proportion of genes which expression changes upon neuronal activity, lost responsiveness after *Tet3* knockdown (Yu et al., 2015). Our transcriptional analyses revealed that synaptic plasticity and memory-related genes are sensitive to changes in TET3 levels. Among the transcriptional target of TET3, we identified the transcription factors CREB1 and ELK1, as well as, the coactivator CREBBP, which are known to play a pivotal role in the formation of long-term memory (Alberini, 2009) via the regulation of immediate early genes such as C-*fos*. Other TET3-sensitive loci include genes involved in active DNA demethylation such as *Gadd45a* and *Gadd45b*. Similar findings were previously reported by Kaas *et al*., who identified genes encoding enzymes that act downstream of TET-mediated 5-mC oxidation, including *Tdg*, *Apobec1*, *Smug1* and *Mbd4*, to be sensitive to TET1 protein levels. Consistently, mapping of TET3 genomic binding sites in the embryonic mouse brain revealed TET3 selective targeting of base excision repair genes (Jin et al., 2016). We further demonstrate that *Notch1* and Notch2 expression levels decrease upon *Tet3* knockdown. Interestingly, previous work has shown that NOTCH signaling is induced in neurons by increased activity, and conditional knockout of *Notch1* in the hippocampus alters synaptic plasticity and memory acquisition (Alberi et al., 2011). In addition, many genes encoding Notch signaling components were previously identified to present activity-induced CpG (de)methylation and expression changes in response to neuronal stimulation in the dentate gyrus (Guo et al., 2011b). TET3 may therefore contribute to the epigenetic control of genes involved in NOTCH signaling pathway upon neuronal activity.

In neuronal cells, TET3 binding was demonstrated to be targeted to genes involved in mRNA processing and splicing, including *Sam68* (Jin et al., 2016). This observation raises the intriguing possibility that *Sam68* is sensitive to TET3 levels. We indeed found that *Sam68* is up-regulated upon *Tet3* knockdown, while its expression is reduced upon *Tet3* overexpression in N2a cells (S11 a, b Fig). Based on these findings, we propose that increased TET3 levels negatively affect *Sam68* gene expression, and this regulatory loop allows TET3 transient expression upon neuronal activity (S12 Fig).

Although specific genes implicated in learning and memory were demonstrated to be susceptible to TET3-mediated transcriptional regulation, little is known about TET3 involvement in memory processes. The only demonstration that TET3 may contribute to memory processes comes from a study by Li *et al.*, which reported that *Tet3* knockdown in the prefrontal cortex is associated with impaired extinction learning (Li et al., 2014b). Further investigations will be required to determine what are the effects of TET3 depletion or overexpression in the hippocampus on memory performance. Furthermore, the effects of miR-29 depletion on 5-mC and 5-hmC profiles and the impact on learning and memory formation remain unknown. Importantly, miR-29a and b have previously been shown to affect synapse formation and plasticity (Lippi et al., 2011) and have been linked to neurodegenerative disease such as Alzheimer's (Hebert et al., 2008). As DNA (de-)methylation is essential for memory formation and plasticity, disrupting the SAM68-miR-29s-TETs regulatory circuit may interfere with physiological functions and contribute to the etiology of neurodegenerative disorders.

3.1 Methods

Animals. C57Bl/6J mice were maintained under a reverse light-dark cycle in a temperature and humidity-controlled facility with food and water *ad libitum*. All experimental manipulations were performed during the animals' active

cycle in accordance with guidelines and regulations of the cantonal veterinary office, Zurich. All behavioral tests were conducted in adult male animals by experimenters blind to treatment.

Contextual fear conditioning. Mice were handled for three days prior to training and testing. Mice were then trained in a contextual fear conditioning (CFC) paradigm (TSE). They were placed in the chamber (context) for 2 mins before receiving three brief electric foot-shocks 1 min apart (0.3 mA for 1s) followed by another 2 min in the chamber. Fear conditioned animals were euthanized 30 min, 3 hours, 24 hours after conditioning. Control animals were exposed to the same chamber for the same duration but received no foot shock and were sacrificed 30 min later. Mice were tested 24 hours after training by re-exposure to the context in the absence of foot-shock. Freezing response was measured for 2 min immediately before and 24h after fear conditioning and was reported as a percentage of time.

Forced swim test. Mice were placed in a small tank of water (18 cm high, 13 cm diameter, 18 ± 1 °C, filled up to 12 cm) for 6 min. Floating duration was scored manually.

Brain tissue collection and processing. Immediately after sacrifice, the brain was removed and the hippocampus rapidly dissected on ice and stored at –80 °C. To avoid potential hemispheric lateralization, both hippocampi were pooled and cryohomogenized as previously described (von Ziegler et al., 2013).

Cell culture. Mouse neuroblastoma (N2a) cells (from ATCC) were cultured in Dulbecco's modified eagle medium (DMEM-high glucose) supplemented with 10% (v/v) fetal bovine serum (Gibco®) and 1% Antibiotic-Antimycotic (Gibco ®). Cells were treated with 40 nM miScript miRNA mimic or inhibitor (Qiagen) and a negative control siRNA with no known target in mammalian genome (All Stars Negative siRNA, Qiagen). Transfections were carried out using lipid-

based HiPerfect transfection reagent (Qiagen). Cells were harvested 24 h after transfection by removing the medium, washing with PBS, and total RNA was isolated using standardized Trizol protocol. Transfection with a pool of siRNAs directed against *Tet3* or *Khdrbs1* (Flexitube Gene Solution, Qiagen) and negative control siRNA (All Stars Negative siRNA, Qiagen) was carried out with Lipofectamine® 2000 transfection reagent (ThermoFischer Scientific) according to the manufacturer's recommendations. In actinomycin D (Tocris®) treatment conditions, the cells were treated with 2.5 *µ*g/ml of the drug prepared in DMSO for 2 h before harvest. Overexpression of *Tet3* were performed using Purefection reagent (System Bioscience) according to the manufacturer's recommendations. Plasmid pEF-DEST51 containing *Tet3* ORF with the CxxC DNA-binding domain was a kind gift from Prof Gerd Pfeifer.

Primary neuronal culture. Neuronal hippocampal cultures were prepared from E-18 embryos and grown in Neurobasal medium supplemented with B27, 1 *µ*g/*µ*l gentamycin, 2 mM glutamax. NMDA stimulation was induced by incubating neurons (11 DIV) for 5 min with 50 *µ*M NMDA, after which neurons were returned to fresh medium. Glycine stimulation was induced in 11 DIV hippocampal cultures as previously described (Raynaud et al., 2013). Briefly, activation of NMDA receptors was achieved by incubating neurons for 3 min with saturating levels (200 µM) of the co-agonist glycine, in Mg^{2+}-free extracellular medium. Neurons were harvested 5 min or 1 h after stimulation in Trizol® reagent (Invitrogen).

miRNA targets prediction. TargetScan6.2 (Lewis et al., 2005), which is based on potential binding site in the 3′ untranslated region of the mRNA and predicted stable thermodynamic binding, was used to predict miRNAs that target *Tet*s. Secondary structures of miR-29s binding sites to *Tet3* 3'-UTR and mimimum free energy were predicted according to RNAfold (Lorenz et al., 2011) or STarMir (Ding et al., 2004).

RNA extraction and real-time quantitative reverse transcription-PCR (RT-qPCR). Mouse hippocampal tissue was homogenized using TissueLyser (Qiagen) in Trizol® reagent (Invitrogen). Total RNA was isolated according to the manufacturer's recommendations. Subcellular fractionation of nuclear and cytoplasmic RNA was performed using Norgen's Cytoplasmic and nuclear RNA purification kit (Norgen BioTek Corp). Nuclear RNA was further treated with RNase-free DNase I kit (Norgen BioTek Corp) to remove genomic DNA contaminations. For mRNAs, total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega). RT-qPCR were performed using SYBR Green (Roche) on a Light-Cycler II 480 (Roche) according to the manufacturer's recommendations. Custom designed gene specific primers were used (S3 Table). Data for brain samples were normalized to two endogenous controls *Gapdh* and *Actb*, and data for cellular samples were normalized to *Tubd1* and *Hprt1*. Cycling conditions: 5 min at 95 °C, 45 cycles with denaturation (10 s at 95 °C), annealing (10 s at 60 °C) and elongation (8-10 s at 72 °C). For miRNAs, total RNA was reverse-transcribed using miScript II reverse transcription kit® (Qiagen). Miscript primer assays for mature and precursor miRNAs (Qiagen) were used to amplify the respective transcripts from a cDNA pool. RT-qPCR was performed in a LightCycler 480 qPCR (Roche) according to the manufacturer's recommendations. Ribosomal *Rnu6* and *Snord61* were used for normalization of Ct values for miRNAs. The primer sequences used for the quantification of mRNAs and miRNAs are shown in Table S3.

Luciferase reporter assays. For validation of *Tet1, 2, 3* and *Dnmt3a* targeting by miR-29b, segments of their 3'UTR including miR-29b seed sequences were amplified from mouse genomic DNA and cloned into pmirGLO Dual-Luciferase miRNA target expression vector (Promega). N2a cells were co-transfected with miR-29b mimics or negative control (All Stars Negative siRNA, Qiagen) and 250 ng of pmirGLO with Lipofectamine® 2000 (Life Technologies) for 24h. Cell extracts were prepared 24h post-transfection, and luciferase activities of firefly and renilla were measured using a Dual-

Luciferase Reporter Assay system (Promega) and with a luminometer GloMax 96 (Promega). Firefly luciferase signals were normalized to Renilla luciferase signals, which serves as internal normalization control. Values were further normalized by that of an empty pmirGLO vector. The primer sequences used for cloning are shown in Table S4.

Statistical Analysis. Statistical comparisons between two groups were performed using an unpaired Student *t*-test. One or two-way ANOVA were performed followed by Dunnett's and Bonferroni's *posthoc* analyses when appropriate. All analyzed data matched the requirements for parametric statistical tests (normal distribution). If variance was not homogenous between groups (determined by Browth-Forsythe's test), adjusted *P* value, *t* value and degree of freedom were determined (Welch correction). Values over two standard deviations away from the mean of each group were considered outliers and excluded from analysis. All statistics were computed with Graphpad Prism. All reported replicates were biological replicates. Significance was set at $P < 0.05$ for all tests. Error bars represent s.e.m. in all figures.

3.2 Acknowledgements

This work was supported by the University of Zürich, the Swiss Federal INsitute of Technology, and the Swiss National Science Foundation. We thank Niharika Gaur for technical help with RT-qPCR assays, Francesca Manuella and Safa Mohanna for assistance with contextual fear conditioning, Giovanna Bosshard and Dubravka Göckeritz-Dujmovic for help with cultured hippocampal neurons, Vanessa Hoop for help with transfection of miR-29b antagomir, Prof Gerd Pfeifer for providing us with the pEF-DEST51-TET3 plasmid, and Ali Jawaid for constructive discussions and helpful comments on the manuscript.

3.3 Authors contribution

EAK carried out contextual fear conditioning paradigm, neuronal stimulation, RT-qPCRs, transfection with miR-29b mimic/antagomir and siRNAs, designed/performed cloning of *Tet* 3'UTRs and luciferase measurements. MAL performed the cloning of *Tet3* and *Dnmt3a* 3'UTR and conducted parts of luciferase assay measurements. JB performed cold swim stress experiments. EAK and IMM designed the study, interpreted the results and wrote the manuscript.

3.4 Supporting information

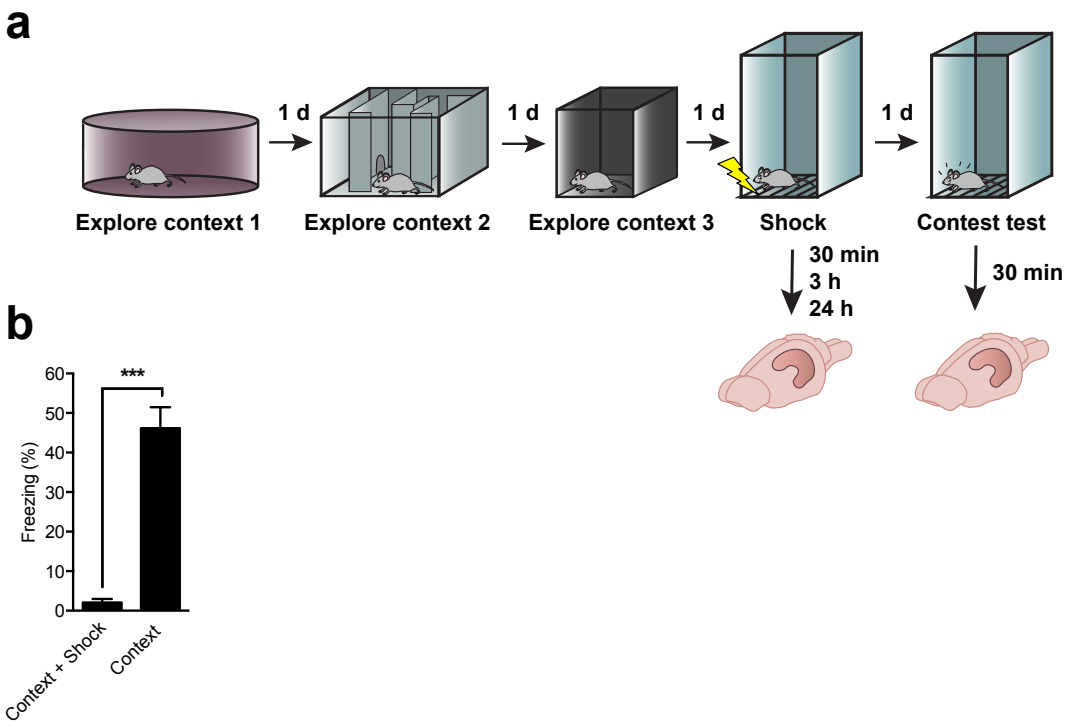

Figure 3-S1 Contextual fear conditioning paradigm. (**a**) During habituation, a mouse was familiarized to an environment different from the home cage (context 1, 2 and 3) and was then placed in a novel context, where it receives 3 electric footshocks. After 24h, the animal was placed back in the context without any shock. The freezing response was measured as an indicator of fear memory. (**b**) Left bar shows baseline freezing before delivery of the foot shock, right bar shows freezing during context test (24h after conditioning). ***p<0.001. Data represent mean s.e.m.

S2 Fig

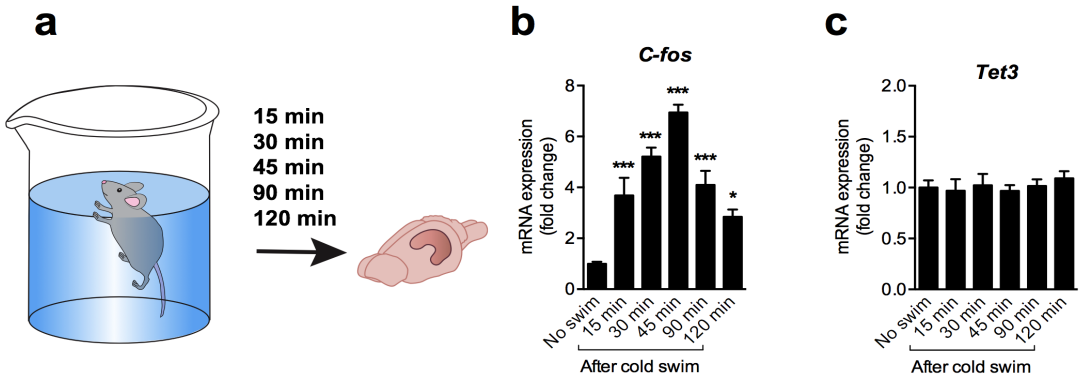

Figure 3-S 2 *Tet3* is not responsive to stress. (**a**) Mice were placed in a small tank of water filled with cold water for 6 min and sacrificed 15, 30, 90, 120 min later (left panel). Level of hippocampal *C-fos* transcripts after subjection to cold swim measured by RT-qPCR. (**b**) Level of hippocampal *Tet1, 2* and *3* transcripts after subjection to cold swim measured by RT-qPCR. Data represent mean s.e.m.

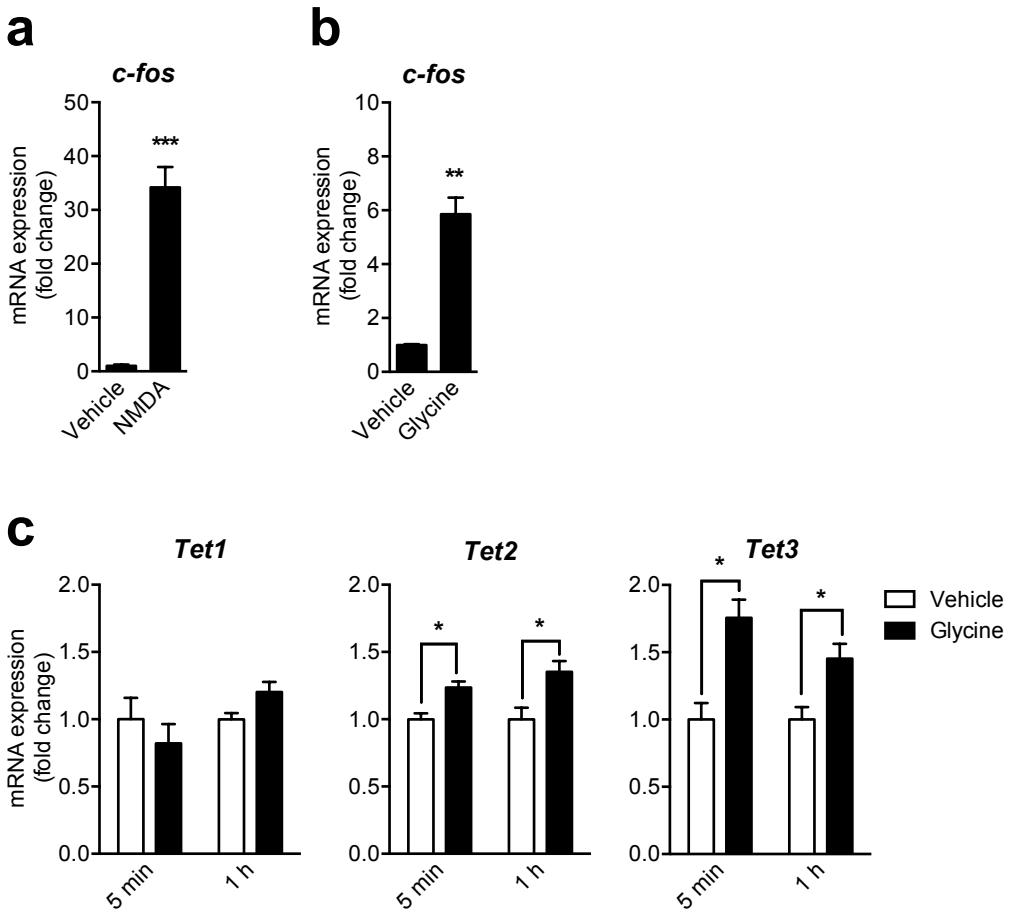

Figure 3-S3 *Tet3* is responsive to activation of NMDA receptors with glycine. (**a**) Level of *C-fos* in hippocampal primary neurons 1h after NMDA stimulation measured by RT-qPCR. (**b**) Level of *C-fos* in hippocampal primary neurons 1h after glycine stimulation measured by RT-qPCR. (**c**) Level of *Tet1*, *2*, and *3* in hippocampal primary neurons 5 min and 1h after glycine stimulation measured by RT-qPCR. *p<0.05. Data represent mean s.e.m.

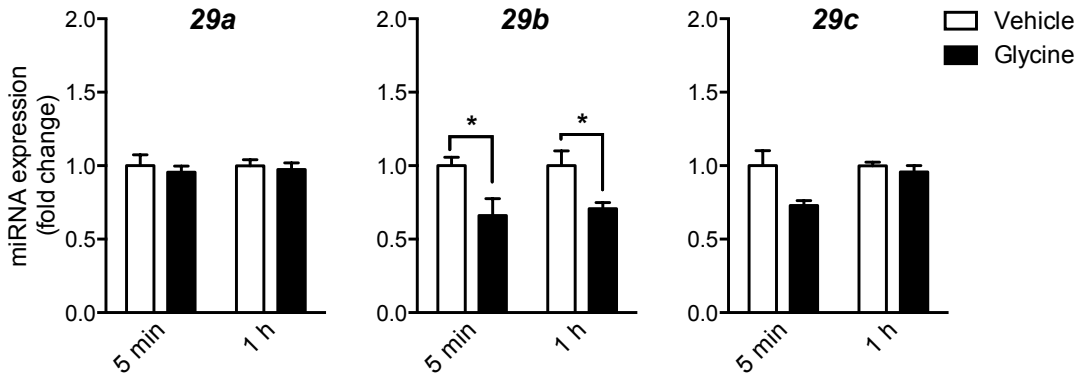

Figure 3-S4 Activation of NMDA receptors with glycine leads to decreased miR-29b expression. Level of *miR-29 a, b,* and *c* in hippocampal primary neurons 5 min and 1h after glycine stimulation measured by RT-qPCR. **p*<0.05. Data represent mean s.e.m.

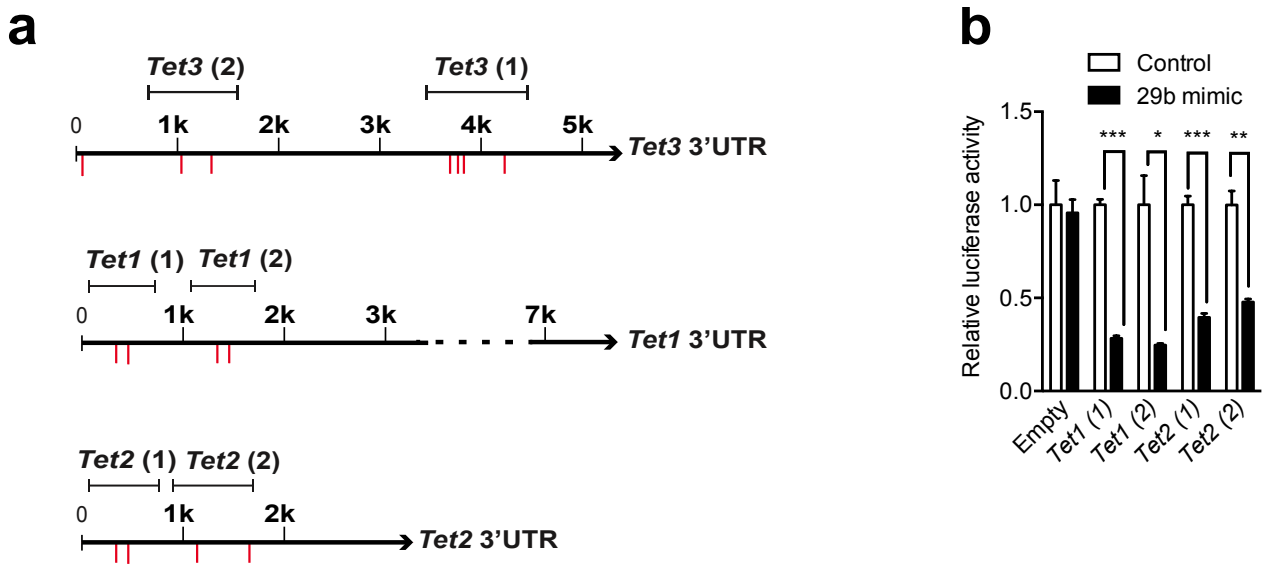

Figure 3-S5 MiR-29b binds to *Tet1 and Tet2* 3' UTRs and control their expression. (**a**) Segments of *Tet1, Tet2 and Tet3* 3'UTRs were cloned into a luciferase reporter; each segment contains conserved seed sequences (indicated in red) for the miR-29 family as predicted by TargetScan [1] (S1 Table). (**b**) Analysis of *Tet1* and *Tet2* luciferase reporters in the presence of miR-29b mimic or control (40 nM) in N2a cells. **p*<0.05, ***p*<0.01, ****p*<0.001. Data represent mean s.e.m

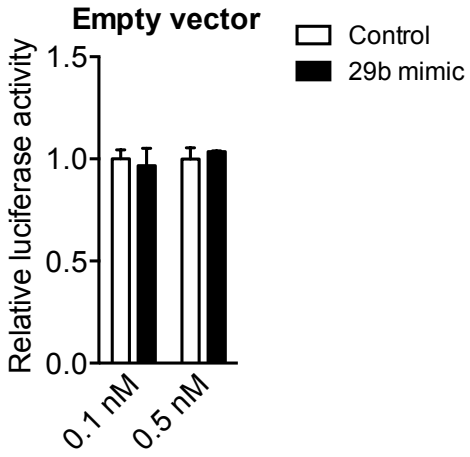

Figure 3-S6 Luciferase activity of empty vector is stable in the presence of miR-29b or control. Analysis of the luciferase activity in N2a cells transfected with empty vector in the presence of graded concentrations of miR-29b mimic or control. Data represent mean s.e.m.

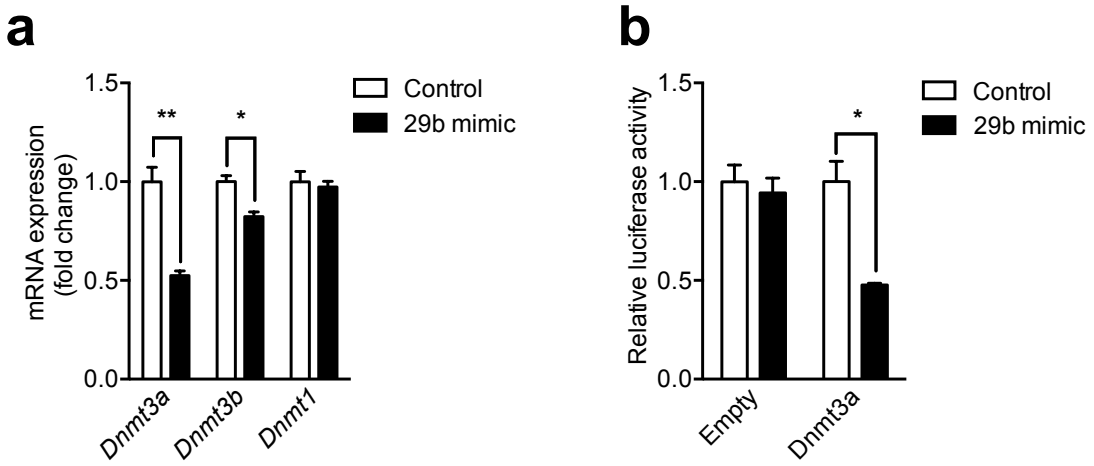

Figure 3-S7 MiR-29b targets *Dnmt3a* and *–b*. (**a**) Level of *Dnmt3a, -b* and *1* in N2a cells 24h after transfection with 29b mimic or control measured by RT-qPCR. (**b**) Analysis of *Dnmt3a* luciferase reporter in the presence of miR-29b mimic or control (40 nM) in N2a cells. **p*<0.05, ***p*<0.01. Data represent mean s.e.m.

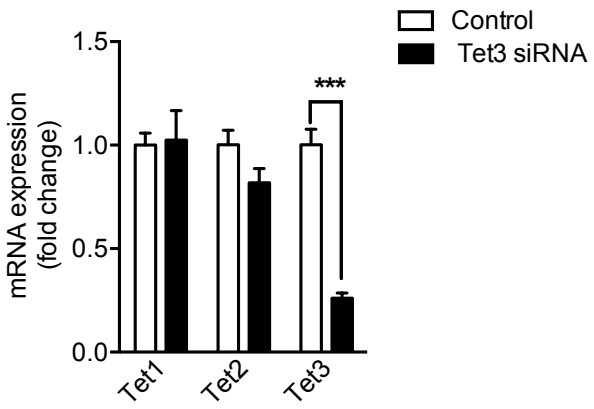

Figure 3-S8 Knockdown of *Tet3* specifically reduces *Tet3* mRNA level. Level of *Tet1*, *Tet2,* and *Tet3* in N2a cells after transfection with a pool of siRNAs directed against *Tet3* or control measured by RT-qPCR. ***$p<0.001$. Data represent mean s.e.m.

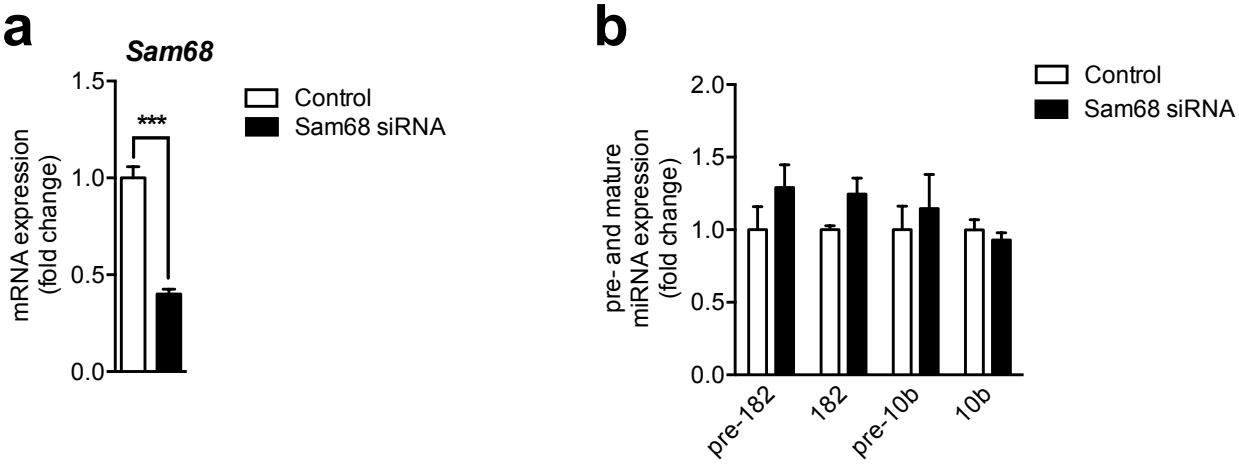

Figure 3-S9 Knockdown of *Sam68* does not alter precursor and mature miR-182 and miR-10b expression level. (**a**) Level of *Sam68* in N2a cells after transfection with a pool of siRNAs directed against *Sam68* or control measured by RT-qPCR. (**b**) Level of precursor and mature miR-182 and miR-10b after transfection with a pool of siRNAs directed to *Sam68* or control measured by RT-qPCR. ***$p<0.001$. Data represent mean s.e.m.

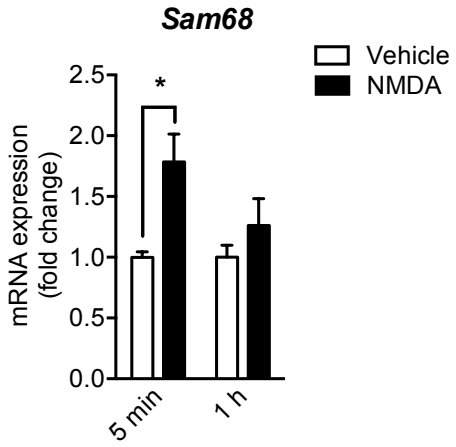

Figure 3-S10 SAM68 modulates the biogenesis of miR-29s at the transcriptional level. Level of pre-miR-29a, b and c in N2a cells after *Sam68* knockdown measured by RT-qPCR in the presence of ActD treatment or vehicle. **p<0.01, ****p*<0.001. Data represent mean s.e.m.

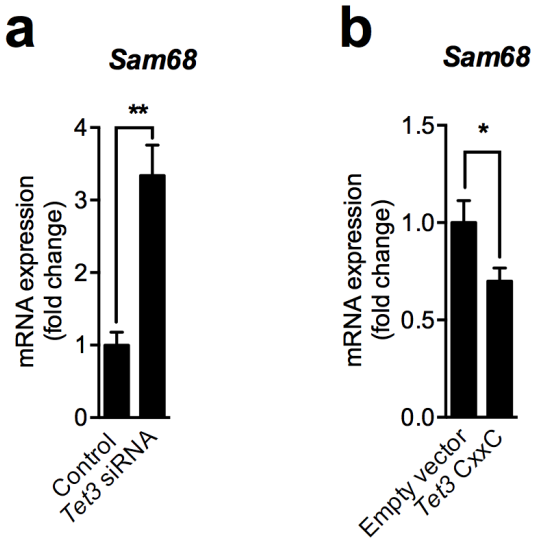

Figure 3-S11 *Sam68* expression is sensitive to TET3 levels. (**a**) Level of *Sam68* in N2a cells after transfection with a pool of siRNAs directed against *Tet3* or control measured by RT-qPCR. (**b**) Level of *Sam68* in N2a cells after transfection with a plasmid overexpressing Tet3 containg the CxxC DNA-binding domain or empty vector measured by RT-qPCR. **p*<0.05, ***p*<0.01. Data represent mean s.e.m.

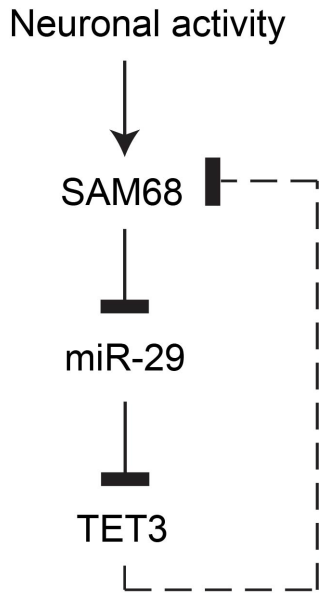

Figure 3-S12 Model for the regulation of *Tet3* via miR-29b and SAM68. Upon neuronal activity, SAM68 inhibits miR-29b transcription, leading to reduced mature miR-29 levels, thus allowing *Tet3* transcripts to increase. TET3 likely inhibits *Sam68* expression by binding to its promoter, thus allowing transient *Tet3* expression in response to neuronal activity.

	Site position	Seed position	Seed type	P_{CT}
Tet3 3'UTR	42-57	51-57	7mer-m8	0.83
	1032-1039	1032-1038	7mer-m8	0.82
	1358-1373	1367-1373	7mer-m8	0.73
	3680-3706	3699-3705	8mer	0.89
	3721-3753	3747-3752	7mer-1A	0.87
	3807-3831	3824-3830	7mer-m8	0.73
	4248-4265	4258-4264	8mer	0.85
Tet1 3'UTR	297-322	316-322	7mer-m8	0.68
	407-423	417-423	7mer-m8	0.89
	1326-1359	1353-1358	7mer-A1	0.7
	1399-1430	1424-1430	7mer-m8	0.89
Tet2 3'UTR	367-405	398-404	7mer-m8	0.68
	525-564	557-563	8mer	0.89
	1032-1061	1055-1061	7mer-m8	0.7
	1587-1621	1614-1620	8mer	0.89

Table S1 Modified output table of TargetScanMouse analysis. List of complementary sites for miR-29s in the 3'UTR region of *Tet1*, *2* and *3* predicted by TargetScanMouse (Lewis et al., 2005). *Tet3* 3'UTR contains seven well-conserved miR-29s binding sites, while *Tet1* and *Tet2* has four only. The site position represents the distance (bp) between the stop codon and binding sites of miR-29b. The seed position represents the distance (bp) between the stop codon and binding sites of the seed sequence of miR-29b. P_{CT} is defined as the probability of conserved targeting.

	mmu-miR-29b-3p				
	Site position	Seed position	ΔG_{hybrid}	Site Access	Seed Access
Tet3 3'UTR	42-57	51-57	-22	0.35	0.447
	963-994	989-994	-18.8	0.365	0.333
	982-1005	1000-1005	-19.5	0.33	0.202
	1010-1016	1010-1015	-16.7	0.294	0.325
	1013-1026	1019-1025	-22.6	0.262	0.17
	1032-1039	1032-1038	-17.5	0.198	0.225
	1148-1164	1159-1164	-21.3	0.477	0.291
	1310-1336	1331-1336	-18.8	0.476	0.314
	1358-1373	1367-1373	-18.4	0.391	0.386
	3062-3082	3077-3082	-21.4	0.455	0.447
	3327-3338	3332-3337	-16.5	0.481	0.291
	3680-3706	3699-3705	-22.9	0.509	0.344
	3721-3753	3747-3752	-20.5	0.58	0.365
	3807-3831	3824-3830	-19.7	0.301	0.286
	4069-4086	4080-4085	-19.3	0.447	0.47
	4178-4201	4195-4201	-20.6	0.452	0.432
	4205-4220	4215-4220	-15.6	0.311	0.406
	4248-4265	4258-4264	-23.1	0.278	0.357
Tet1 3'UTR	6454-6490	6483-6489	-25.7	0.346	0.458
	407-423	417-423	-20.4	0.306	0.409
	79-96	90-95	-18.3	0.372	0.241
	2043-2082	2076-2082	-19.1	0.338	0.253
	297-322	316-322	-18.1	0.453	0.555
	1326-1359	1353-1358	-21.5	0.304	0.49
	1521-1537	1532-1537	-19	0.44	0.178
	1399-1430	1424-1430	-21.7	0.412	0.854
	5250-5275	5269-5275	-22.5	0.464	0.446
	6987-7004	6999-7004	-20.6	0.316	0.18
	4551-4581	4574-4580	-24.5	0.241	0.041
	457-474	469-474	-20.5	0.193	0.204
	7651-7665	7660-7665	-16.2	0.154	0.032
	1455-1476	1471-1476	-17.9	0.305	0.144
Tet2 3'UTR	1587-1621	1614-1620	-23.3	0.375	0.13
	525-564	557-563	-28.6	0.417	0.463
	1032-1061	1055-1061	-19	0.465	0.456
	367-405	398-404	-25	0.203	0.268
	1129-1168	1161-1167	-20.8	0.333	0.07

Table S2 Modified output table of STarMiR analysis. Free energy in kcal/mol (ΔGhybrid) analysis of putative miR-29b binding site to each *Tet* 3'UTR, and measure of the structural accessibility in the predicted binding site (Site Access) or in the target sub-region complementary to the miRNA seed (Seed Access) as defined by STarMir (Ding et al., 2004). Highlighted rows are conserved miR-29b binding sites as predicted by TargetScanMouse (Lewis et al., 2005) (S1 Table).

Genes	Forward primer (5'->3')	Reverse primer (5'->3')
Tubd1	TCTCTTGCTAACTTGGTGGTCCTC	GCTGGGTCTTTAAATCCCTCTACG
Hprt1	GTTGGGCTTACCTCACTGCTTTC	CCTGGTTCATCATCGCTAATCACG
Actb	CAACGGCTCCGGCATGTGC	CTCTTGCTCTGGGCCTCG
Gapdh	CAGCAATGCATCCTGCACC	TGGACTGTGGTCATGAGCCC
Tet1	TTGCTGGAGACTGTCGACTTGG	TGCTCGAATCAACGTACACACCAC
Tet2	TGCCAAATGGCAGTACAGTGGTG	ATCCTCAGGCTTAGCTCCGACTTC
Tet3	GCATCGGGCAGGCCACCATT	GGCAAGCACAGGTCCGGTCA
Dnmt3a	CAGCTGCTTACGCCCCACCC	CACCAGCCGCTCCCTTGTGC
Dnmt3b	AAAGCCCGGCTGTCCGAACC	CCCTGCCGACCTCGGGTGAT
Dnmt1	AGTCTGTTCCTGTGCAGAAGGC	TGCTGAAGAAGCCATCCCACTC
Fos	ACAGATACACTCCAAGCGGAGAC	TGGCAATCTCAGTCTGCAACGC
Drosha	CATCACGAAGGACACTTGACGTTG	TGCTACCTTGGCTTGCGTTCTG
Dgcr8	GTCACTTGGTCCAGACCCTACTTC	GCTTAGAGGAGGATCATGTTTCCG
Dicer	TCTTCGAG CTCCATTGTTGGTC	CTACCACTCTTTCACCAACCG
Ddx5	ACCATTGACGCCATGTCGAG	CAAATCGAGGTGCACCAAACCC
Ddx17	AGGGATATGGTTGGCATTGCACAG	CAATCGCAGGCAGCAAATACGC
p53	CACGTACTCTCCTCCCCTCAAT	AACTGCACAGGGCACGTCTT
Notch 1	ACAGTGCAACCCCCTGTATG	TCTAGGCCATCCCACTCACA
Notch 2	ACAGTGTTGGCTCCCTGTTC	ATCGTTTACCTTGCCAGCCA
Khdrbs1	TTATGGCCCATGCTATGGAAGA	AGGTACTCCGTTCAAGTAGGAC
Elk1	CTGCTCCCCACACATACCTT	GAGAGGCCATCCACACTGAT
Elk4	ATCTAACAATGGGGAGTTCAAGC	GGCTCGGCTGAGTTTATCATAAT
Gadd45a	TGC GAG AAC GAC ATC AAC AT	TCC CGG CAA AAA CAA ATA AG
Gadd45b	CTGCCTCCTGGTCACGAA	TTGCCTCTGCTCTCTTCACA
Tdg	TAGGAAACGTGCGTGTTCAG	CTCATACTGCCAAACCAGCA
Crebbp	TGGAGTGAACCCCCAGTTAG	TTGCTTGCTCTCGTCTCTGA
Creb1	AGCTGCCACTCAGCCGGGTA	TCGCCTGAGGCAGCTTGAACA
miRNAs	**Source**	
miR-29b-1	Qiagen, cat. No.: MS00005936	
miR-29a	Qiagen, cat. No.: MS00001372	
miR-29c	Qiagen, cat. No.: MS00001379	
miR-10b	Qiagen, cat. No.: MS00032249	
miR-182	Qiagen, cat. No.: MS00011291	
Snord61_11	Qiagen, cat. No.:MS00033705	
Rnu6	Qiagen, cat. No.: MS00033740	
Pre-miR-29b-1	Qiagen, cat. No.: MP00005355	
Pre-miR-29a	Qiagen, cat. No.: MP00005348	
Pre-miR-29c	Qiagen, cat. No.: MP00005369	
Pre-miR-10b	Qiagen, cat. No.: MP00003983	
Pre-miR-182	Qiagen, cat. No.: MP00004431	
Pri-miR-29a/b-1	AACTATTGCACGGACTTCACCT	TCCTGA AGA AGCTTTGTCGTC

Table S3 List or primers used for the quantification of mRNA and miRNA transcripts.

Name	Forward primer (5'->3')	Reverse primer (5'->3')
Tet1 (1)	AATGCCTTTGCTAATGTGGTG	TTAGCGAACAGCTTCCAACC
Tet1 (2)	AGGAAAATGGGAACCCAAAC	TGAGGGAGGATTTCTGATGG
Tet2 (1)	AATGCCTTTGCTAATGTGGTG	TTAGCGAACAGCTTCCAACC
Tet2 (2)	TCGGCTGATGAGCAGTATCA	AGCAATCTGGGTAGCACCAT
Tet3 (1)	TTTAAAGAAACAGTAGTTTGCAGAGC	TATCATACCCTCATGGAATCTAAGTT
Tet3 (2)	GCTCTTCTCGTCCCGTTGAT	TAGAGCCACGTGCTAACTGC
Dnmt3a	TTGGCCTTGCAAAAGGGTTG	TTGCACGCGAGTCTGGATAA

Table S4 List or primers used for cloning

4 Discussion and outlook

The exciting discovery of TET proteins provided a mechanism by which DNA demethylation may occur as they contribute to the removal of 5-methylcytosine (5mC) by converting it to 5-hydroxymethylcytosine (5hmC). Given that 5hmC and TETs are abundant in the central nervous system, much effort has been made to understand their roles in neuronal function.

While preparing the manuscript presented in Chapter 3, two major studies reported that TET3 in the brain functions as an important regulator of neuronal activity and memory. Yu and colleagues confirmed that *Tet3*, but not *Tet1* and *Tet2,* is regulated at the mRNA level by neuronal activity in cultured hippocampal neurons. They further identified TET3 as an important player in the maintenance of homeostatic synaptic plasticity. At the molecular level, TET3 appears to elicit these effects through the regulation of the surface level of the AMPA receptor subunit GluR1 (Yu et al., 2015). These findings provided additional evidence that neuronal activity can modulate TET3 expression and function *in vitro*.

Similarly, Li et al. demonstrated that *Tet3* expression is up-regulated in cultured cortical neurons in response to neuronal depolarization using potassium chloride. In their study, extinction training led to an increase in *Tet3* mRNA in the infralimbic prefrontal cortex (IlPFC), a brain region essential for fear memory extinction (Li et al., 2014b).

The present study is complementary and builds upon these two studies. We provide evidence that *Tet3* is regulated by learning in the hippocampus, a region related to cognitive processes in the brain. Furthermore, we extend their findings by investigating the up-stream mechanism controlling *Tet3* expression.

4.1 TET3 expression is activity-dependent

Evidence from our study, as well as, the above-mentioned independent analyses establish that *Tet3* expression is regulated by neuronal activity *in vitro* and *in vivo.* Treatment with tetrodotoxin, a sodium channel blocker and inhibitor of synaptic activity, or bicuculline, a GABAA receptor antagonist and promoter of synaptic activity, decreases and increases *Tet3* expression, respectively (Yu et al., 2015). These findings demonstrate that *Tet3* is modulated bi-directionally. They also confirm the effect at the protein level but only after 4 h of continuous pharmacological treatment. In our case, brief application of NMDA, an agonist of NMDA receptors that mimics the action of the neurotransmitter glutamate, was sufficient to trigger a rise in *Tet3* transcripts in hippocampal neurons. Further, Li *et al.* observed that pharmacological inhibition of NMDA receptors activity *in vivo* blocks the increase in *Tet3* expression in the IIPFC associated with fear memory extinction (Li et al., 2014b). These findings collectively suggest that neuronal activity induces an increase in *Tet3* level via an NMDA receptor-dependent signaling pathway.

The observation that *Tet1* is unaltered in the hippocampus upon contextual fear conditioning (CFC) appears, at first glance, intriguing as an earlier report by Kaas *et al.* showed that *Tet1* but not *Tet3* is regulated by activity (Kaas et al., 2013). In this study, however, TET enzymes were specifically quantified in the CA1 area of the dorsal hippocampus, a hippocampal subregion that has been associated with retrieval of contextual memories (Daumas et al., 2005; Lee and Kesner, 2004). As the hippocampus is composed of multiple subregions, including CA2, CA3 and dentate gyrus, TET3 could be differentially modulated by neuronal activity in different hippocampal regions.

Similarly, behavioral experiences were reported to alter the expression of other components of the DNA (de)methylation machinery, such as *Dnmt3a* (Miller and Sweatt, 2007), *Dnmt3a2* (Oliveira et al., 2012), and *Gadd45b*

(Sultan et al., 2012). In particular, the expression of *Dnmt3a2* in the hippocampus is robustly and transiently enhanced by neuronal activity *in vitro* and by learning *in vivo*, and this modulation depends on the activation of NMDA receptors. Interestingly, these changes occur during the initial consolidation window (shortly post-training, within 3 h) in the hippocampus in response to training. This indicates that they are important for memory acquisition, rather than for the persistence of memory over time.

We observed that *Tet3* expression goes back to baseline level 24 h after training; a time-point at which a second wave of hippocampal gene expression occurs that is required for the persistence of long-term memory (LTM) (Katche et al., 2010). Consistently, Miller and Sweatt have reported a similar temporal pattern of DNMT gene expression and DNA methylation in the hippocampus following CFC. Accordingly, locus-specific DNA methylation occurred 1 h after training but these changes in DNA methylation returned to baseline within 24 h (Miller and Sweatt, 2007). Other epigenetic processes, such as histone acetylation, histone methylation, and histone phosphorylation exhibit similar temporal regulation pattern in the hippocampus after fear conditioning (Chwang et al., 2006; Gupta-agarwal et al., 2012; Gupta et al., 2010; Levenson et al., 2004).

Our results further indicate a non-significant increase in hippocampal *Tet3* transcripts on memory reactivation (memory retrieval). In line with this finding, a recent study revealed an increase in global 5hmC levels in the CA1 region of the hippocampus in rats, as well as, *Tet3* mRNA and protein levels 1 h after the reactivation of fear memory (Webb et al., 2017). To date, very little is known about the extent to which epigenetic mechanisms contribute to memory retrieval and further studies are required to elucidate these mechanisms. In summary, epigenetic modifiers, including TETs, seem to occur in waves in the hippocampus, thus they may contribute to the temporal regulation of the gene expression profile that is critical for memory.

Although *Tet3* gene expression was induced by activity in the hippocampus and in cultured neurons, we could not confirm the effect at the protein level. Unfortunately, commercially available anti-TET3 antibodies are highly unreliable. In addition, at least three different TET3 isoforms are known to exist: an isoform containing an N-terminal CXXC domain, one isoform lacking the CXXC domain, and finally one isoform that is oocyte-specific (Jin et al., 2016). Neuronal activity may enhance the expression of distinct isoforms that are not detectable with commercially available antibodies.

Another possibility is that TET3 protein may have a very high turnover rate in neurons, making it difficult to detect. Interestingly, a recent study provided evidence that TET proteins are direct substrates of calpains, a group of calcium-dependent proteases (Wang and Zhang, 2014). As neuronal activity triggers calcium-signaling cascades in neurons, studying how calpain-mediated TET degradation in the context of neuronal activity could be pertinent.

Although TET3 expression is dynamically regulated in the hippocampus upon fear memory acquisition and retrieval, as well as, in the prefrontal cortex after memory extinction, little is known about the function of TET3 in these processes. The strongest evidence was provided by Li and colleagues, who showed that lentiviral-induced TET3 reduction in the prefrontal cortex is sufficient to impair fear memory extinction (Li et al., 2014b). Further research will be required to determine what are the effects of TET3 depletion or overexpression in the hippocampus on memory performance. As deletion of *Tet3* leads to neonatal lethality (Gu et al., 2011), conditional brain specific *Tet3* knockout models would be most suitable for such analysis. A further consideration while designing these studies would be to account for the redundancy and compensatory functions between different TET enzymes (Dawlaty et al., 2013).

4.2 TET3 acts as a regulator of gene expression

We postulated that activity-dependent Tet3 up-regulation in the hippocampus upon CFC affects the transcriptional activity of genes related to the formation of contextual memories, likely by modulating the 5mC and 5hmC landscape of its target. The idea that TET3 plays a role in transcriptional regulation is also supported by the observation that TET3 interacts with transcriptional regulators and histone writers such as the RE1-silencing transcription factor (REST) and histone-lysine N-methyltransferase (NSD3) in neurons (Perera et al., 2015).

Using siRNA-mediated *Tet3* knockdown *in vitro*, we identified altered expression of genes coding for transcription factors and their coactivators, such as *Creb1*, *Elk1* and *Crebbp*, which are crucial for the formation of memory (Alberini, 2009). Furthermore, expression level of genes coding for effectors of the active DNA demethylation pathway, including *Gadd45a* and *Gadd45b* is altered upon *Tet3* knockdown conditions. Importantly, neuronal activity has been shown to alter *Gadd45b* expression, which mediates gene-specific demethylation of *Bdnf*, a gene coding for a growth factor important for neurogenesis, synaptic plasticity, and learning and memory (Ma et al., 2009; Sultan et al., 2012). TET3 may therefore be implicated in the regulation of *Gadd45b* in the context of neuronal activity.

Lastly, we showed that *Notch1* and *Notch2* are sensitive to *Tet3* levels. Notch signaling components, including *Notch 1* and *Notch 2*, have been previously associated with activity-induced CpG (de)methylation and expression changes in response to neuronal stimulation in the rodent dentate gyrus (Guo et al., 2011b), which points towards a role of TET3 in the activity-dependent regulation of *Notch* genes in the context of neuronal activity, However, further experiments validating these *in vitro* findings in the brain are needed.

Meanwhile, other studies have employed high-throughput approaches in order to examine the impact of TET3 manipulation on transcription regulation and its effect on neuronal morphology and physiology. Specifically, overexpression of TET3 in mouse olfactory sensory neurons (mOSN) altered 5hmC patterns and expression of mOSN-specific genes (Colquitt et al., 2013). In addition, lentiviral-mediated TET3 overexpression in retinal explant cultures resulted in the up-regulation of 981 proteins, mainly related to neuronal functions (Perera et al., 2015). NOTCH1 and 2 were listed among the 981 up-regulated proteins by TET3 overexpression, confirming our earlier observation. Overall, these findings are indicative of a role for TET3 in regulating gene expression in the brain.

To dissect the role of TET3 in synaptic activity-dependent gene expression, Yu and coworkers assessed global gene expression changes in *Tet3* knockdown neurons in the presence and absence of neuronal activity. *Tet3* knockdown resulted in the differential expression of 2873 genes, mainly genes involved in synaptic transmission. Interestingly, 85% of the activity-dependent genes that exhibited differential expression in response to neuronal activity lost responsiveness in *Tet3* knockdown neurons (Yu et al., 2015). This means that TET3 is an essential regulator of activity-dependent gene transcription in neurons. *In vivo* studies are required to validate these findings in the brain.

Finally, for confirmation of TET3 targets, chromatin-immunoprecipitation is an ideal approach. This technique, however, has been difficult to implement owing to the lack of chromatin immunoprecipitation (ChIP)-grade TET3 antibodies. To circumvent this problem, Yu and colleagues took advantage of a FLAG-tagged TET3, and demonstrated a direct binding TET3 to *Bdnf* promoter region in hippocampal neurons (Yu et al., 2015). This is of particular interest as *Bdnf* promoter is susceptible to demethylation after neuronal stimulation (Ma et al., 2009).

Very recently, endogenous TET3 was mapped across the whole genome in mouse embryonic brain (E15.5) using a homemade antibody specifically targeting TET3 DNA-binding domain CXXC. Although restricted to one specific TET3 isoform, this is the first study that provides information about TET3 genomic localization in the brain. TET3 appears to be targeted near transcription start sites of genes involved in lysosome function, mRNA processing, splicing, and base excision repair (Jin et al., 2016). The association of TET3 at genes functioning in base excision repair is intriguing. TET proteins seem to share redundant functions as manipulation of TET1 in the brain affects the expression of genes related to TET-mediated 5mC oxidation such as *Tdg*, *Apobec1*, *Smug1* and *Mbd4* (Kaas et al., 2013). Furthermore, it was reported that TET1 interacts with TDG and stabilizes its activity, providing strong evidence for a coupling of 5mC oxidation and TDG-initiated base excision repair (Weber et al., 2016).

4.3 MicroRNA-29s, a family of epi-miRNAs

MiRNAs are critical regulators of gene expression in the brain, performing post-transcriptional regulation of gene expression in a rapid and site-specific manner in response to neuronal activity (Sim et al., 2014). We demonstrate that miR-29b binds to the three prime untranslated region (3'UTR) of all *Tets* and regulates their expression. Importantly, even low amounts of miR-29b can regulate *Tet3* and to a lesser extent *Tet2,* while a higher amount affects all *Tets*. Consistently, other studies reported that the three members of the miR-29 cluster target the 3'UTR of *Tet1* in human cancer cell lines (Morita et al., 2013; Zhang et al., 2013a). Interestingly, they can also modulate *Tdg* transcript levels, indicative of a general role for this miR cluster in the regulation of components of the DNA demethylation machinery (Morita et al., 2013).

Tet 3'UTRs contain predicted binding sites for other miRNAs, many of which are altered by neuronal activity and learning, including miR-140 and miR-291 (Kye et al., 2011). However, 3'UTR of *Tets* has the highest number of predicted binding sites for 29s according to *in silico* analyses provided by Targetscan, which suggests that *Tet* transcripts are likely to be more sensitive to miR-29s as compared to any other miRNAs. Indeed, we observed that the expression of the miR-29 cluster is altered upon neuronal activity *in vitro* and by learning *in vivo*, in a manner opposite to *Tet3*.

Independent analyses have previously suggested a sensitivity of miR-29 family to neuronal activity and its potential role in learning and memory. Kye et al. examined the expression profile of selected miRNAs upon CFC in the hippocampal CA1 region. Among many other miRNAs, miR-29b expression level was significantly decreased 1 h after training, consistent with our results (Kye et al., 2011). Furthermore, high-throughput profiling of miRNA expression in the hippocampus in response to neuronal activity revealed that only a subset of miRNAs is induced by activity while nearly all miRNAs decline in expression (Eacker et al., 2011) Therefore, a decline in the level of mature miRNAs, such as for miR-29b, likely contributes in establishing a permissive landscape for *de novo* protein synthesis. In particular, if simultaneously the miRNA target's abundance rises enough to titrate the miRNA, then all targets of that miRNA should be derepressed as suggested previously (Mukherji et al., 2011). Therefore, if the pool of *Tet1, 2* and *3* mRNA is below the saturation regime of miR-29b, then all *Tets* will be similarly repressed regardless of expression level. However, as *Tet3* mRNA level raises following neuronal activity, target de-repression due to miR-29b saturation might occur. It remains to be determined, however, whether miR-29b decline is essential for *Tet3* activity-dependent up-regulation after neuronal activity.

4.1 SAM68 regulates miR-29 biogenesis

MiRNA biogenesis is a tightly regulated process, with complex mechanisms controlling the transcriptional production of pri-miRNAs, as well as, their subsequent processing by the microprocessor complex and DICER (Loffreda et al., 2015). Importantly, some RNA-binding proteins (RBPs) have been implicated in these regulatory processes. We found that the RBP SAM68 (Src-associated in mitosis 68 kDa protein), also known as KHDRBS1 (KH domain containing, RNA binding, signal transduction associated 1) is involved in the biogenesis of the miR-29 cluster.

SAM68 belongs to the STAR (signal transduction activator of RNA metabolism) family of RBPs, which regulates various aspects of RNA metabolism in response to signaling cascades, including transcription, pre-mRNA splicing and RNA transport. SAM68 regulates splicing during cell differentiation, namely upon spermatogenesis, adipogenesis, and neurogenesis (Paronetto et al., 2011; Vogel and Richard, 2012). In addition, SAM68 regulates the activity-dependent alternative splicing of Neurexin-1 in the central nervous system (CNS), a critical regulator of synaptogenesis (Iijima et al., 2011). Notably, previous studies have documented a role of several splicing factors in the biogenesis of selected miRNAs. For example, the splicing factor hnRNPA1 has been implicated in the production of miR-18a (Guil and Caceres, 2007) Similarly, the KH-type splicing regulatory protein (KSRP) has been shown to promote the biogenesis of only a subset of miRNAs, including let-7, by interacting with both DROSHA and DICER (Trabucchi et al., 2009). In this respect, SAM68 has been shown to play a role in the miRNA processing in chromatoid bodies during spermatogenesis (Messina et al., 2012). Notably, twelve miRNAs were found to be differentially expressed in wild type and *Sam68* knockout germ cells, including miR-29b. Further, SAM68 interacts with both DROSHA and DICER in male germ cells, in a RNA-independent manner, suggesting a role of SAM68 in miRNA biogenesis.

Our results indicate SAM68 likely exerts its regulatory function at the transcriptional level. In line with our results, previous studies have implicated SAM68 in transcriptional regulation, mainly by acting as a competitive inhibitor of transcriptional activators. For example, interaction of SAM68 with hnRNPK, a RNA-binding protein involved in pre-mRNA processing, leads to the inhibition of hnRNPK function as a transcriptional activator (Yang et al., 2002). Additionally, SAM68 itself was identified as a potent transcriptional repressor that is independent of its RNA binding ability (Hong et al., 2002). SAM68 was further described to functionally interact with the CREB-binding protein (CBP), and negatively modulates its transcriptional activity. Finally, SAM68 has been shown to directly interact with RNA polymerase II in meiotic spermatocytes (Paronetto et al., 2011). Therefore, SAM68 binding to transcriptional coregulators and to RNA polymerase II itself points towards a role of SAM68 in transcriptional regulation of gene expression.

In line with this, we provide evidence that SAM68 negatively regulates the biogenesis of the miR-29 cluster, likely at the transcriptional level, as SAM68 knockdown results in the up-regulation of primary miR-29a/b, as well as, precursor and mature miR-29b transcripts. Based on the aforementioned ability of SAM68 to interact with specific transcriptional regulators and modulate their activity, it is likely that SAM68 negatively regulates miR-29 transcript levels by functionally interacting with miR-29 transcriptional regulators. A CCAAT-enhancer binding protein (C/EBP) binding site has been confirmed immediately downstream of the miR-29b-1/a transcription start site (TSS) (Eyholzer et al., 2010). This transcription factor is known to recruit CBP (Kovacs et al., 2003), an acetyl-transferase whose transcriptional activity can be modulated by SAM68 (Hong et al., 2002). Similarly, three nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) binding sites have been characterized in the human miR-29b-1/a promoter located at -561, -110, and +134 relative to the TSS (Mott et al., 2011). Phosphorylated SAM68 has been shown to associate with NF-KB complexes in T-cell lymphoma cell line,

and facilitate binding to the NF-KB consensus motif (Fu et al., 2013). It is therefore likely that SAM68 mediates miR-29b-1/a transcriptional regulation via interaction with CBP and/or NF-KB.

Importantly, SAM68 has the ability to link signal transduction pathways to downstream processes in response to phosphorylation and other post-transcriptional modifications (Najib et al., 2005). Notably, SAM68 possesses a C-terminal domain rich in tyrosine residues, which are potential substrates for tyrosine kinases (Di Fruscio et al., 1999). For example, SAM68 can be phosphorylated by the extracellular signal-regulated kinase 1 (Erk1) (Matter et al., 2002), and lysine acetylated by CBP (Babic et al., 2004). Consistently, neuronal activity was found to induce SAM68 phosphorylation at serine 20 (Iijima et al., 2011). This is highly relevant; especially as activity-dependent neuronal signal transduction is critical for the regulation of gene expression associated with LTM. Therefore, SAM68 function is likely to be modulated by phosphorylation upon neuronal activity.

Therefore, SAM68 represents a key candidate factor for the activity-dependent regulation of miR-29b owing to its potential to link neuronal to miR-29b transcriptional regulation. Additional experiments are however required to validate this hypothesis. An important experiment in this regard could be checking miR-29b biogenesis and *Tet3* gene expression after stimulation of hippocampal neurons from SAM68 knockdown mice. Furthermore, it would be of great interest to examine the role of SAM68 in cognitive processes. SAM68 is abundantly expressed in different brain regions, such as the hippocampus, the cortex and the cerebellum (Grange et al., 2004; Iijima et al., 2011). However, very little is known regarding its function within the CNS. Interestingly, *Sam68* knockout mice were shown to exhibit fewer spines on the dendrites of CA1 pyramidal neurons in the hippocampus (Klein et al., 2013). Consistently, miR-29b overexpression reduces spine density in cultured hippocampal neurons (Lippi et al., 2011), suggesting that SAM68 may be involved in dendritic spine structure via regulation of miR-29b. As

there is a strong interaction between dendritic spine density and memory acquisition in the hippocampus, SAM68 and miR-29b are likely involved in memory processes.

Most importantly, we found that *Sam68* is sensitive to TET3 levels as suggested by knockdown and overexpression experiments. This agrees with the observation that TET3 binding is targeted to genes involved in mRNA processing and splicing in neuronal cells (Jin et al., 2016). Importantly, *Sam68* was included in the list provided by the authors describing TET3 binding sites as revealed by ChIP sequencing. In addition, we observed that *Sam68* exhibits an expression pattern upon CFC opposite to *Tet3* (data not shown). Based on these findings, we propose that increased TET3 levels negatively affect *Sam68* gene expression, and this regulatory loop allows *Tet3* transient expression upon neuronal activity.

4.2 Conclusion

Overall, these findings newly reveal the existence of a molecular cascade that involves the miR-29 cluster and the RNA-binding protein SAM68 in the regulation of the DNA demethylase TET3 (Fig. 4-1).

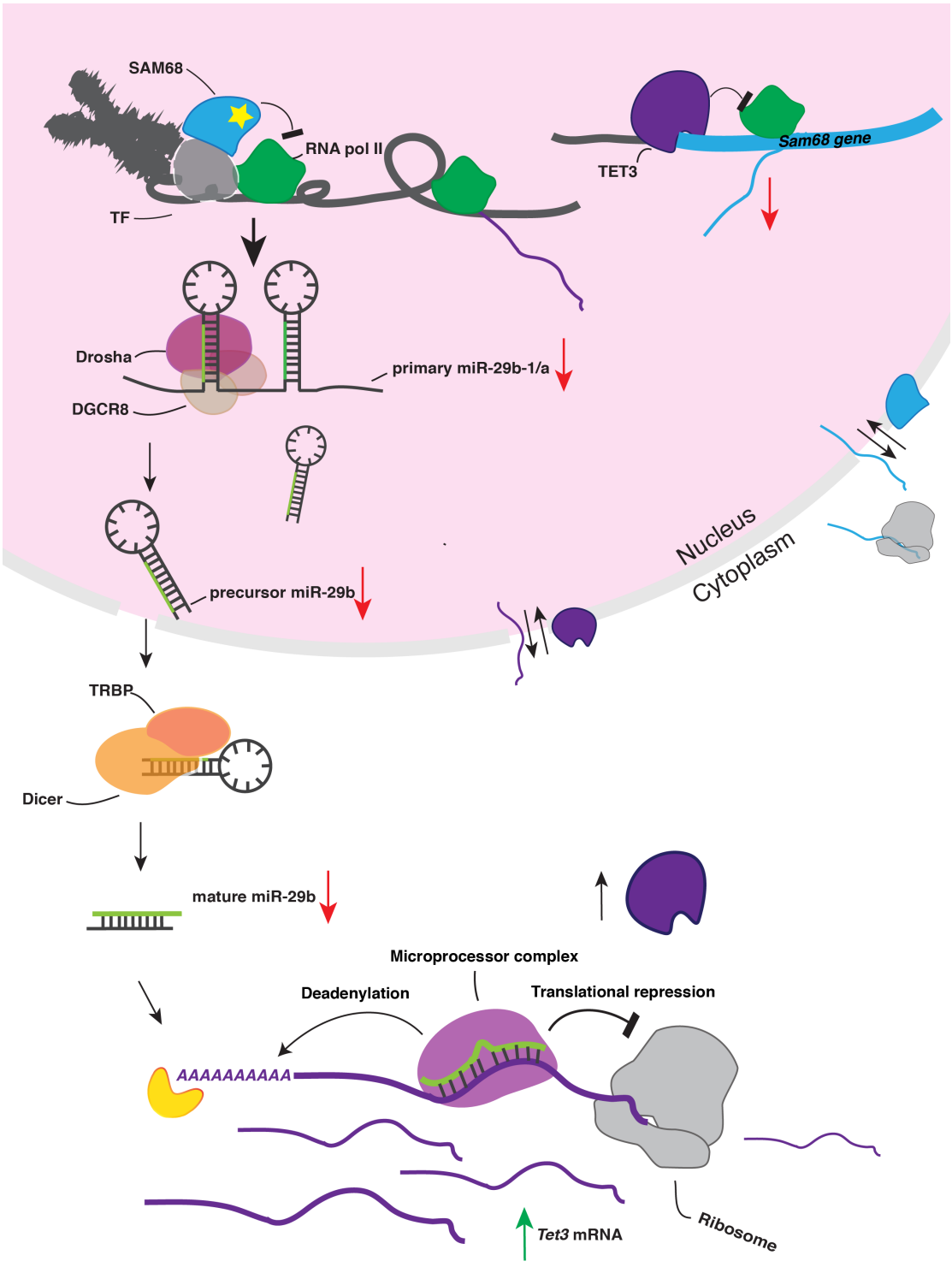

Figure 4-1 Model for the regulation of *Tet3* upon neuronal activity by its targeting miRNA, miR-29b and the RNA-binding protein SAM68. SAM68 negatively modulates the transcriptional activity of the primary transcript miR-29b-1/a (refer to Fig. 3-5), which ultimately leads to decreased expression in miR-29b levels (Fig. 3-2). Reduced level in miR-29b correlates with increased *Tet3* levels (Fig. 3-1). MiR-29b likely facilitates *Tet3* translation or fine-tunes its gene expression as it is able to binds to its 3'UTR and control its expression (Fig. 3-3). TET3 inhibits *Sam68* expression (Fig. 3-S11) suggesting the existence of a feedback loop to allow *Tet3* transient expression upon neuronal activity.

We propose a model by which SAM68 negatively regulates the transcriptional activity of miR-29b, likely via the interaction with transcription factors, which leads to alterations in miR-29b biogenesis. As a consequence, the expression of miR-29b-1/a primary transcripts is reduced, as well as, precursors and mature miR-29b. Decline in miR-29b expression creates a permissive environment for *Tet3* translation and/or fine-tunes *Tet3* gene expression. Consequently, increased *Tet3* expression modulates activity-dependent gene transcription of targets related to synaptic plasticity and memory, such as *Creb1* and *Notch2*. In contrast, the expression of *Sam68*, another target of TET3, is repressed, which keep *Tet3* expression transient.

The identification of molecular steps through which SAM68 regulates miR-29 and TET3 are not only relevant for neuroscience but also for translational medicine. Consistently, loss of SAM68 function has been associated with the pathogenesis of the fragile X tremor/ataxia syndrome (FXTAS), a late onset neurodegenerative disorder characterized by ataxia and cognitive decline (Sellier et al., 2010). Consistently, *Sam68* knockout mice have been reported to present motor-coordination deficits (Lukong and Richard, 2008). Interestingly, miRNA processing was reduced in patients with FXTAS (Sellier et al., 2013), and brain-specific knockdown of miR-29b resulted in an ataxic phenotype in mice (Roshan et al., 2014). This suggests that dysregulation of SAM68, thus miR-29b, could contribute to the pathogenesis of FXTAS. It would be of great interest to investigate whether or not TET3 expression and function are altered in FXTAS. In line with this idea, genome-wide alterations of 5hmC have been observed in the cerebellum of a FXTAS mouse model (Yao et al., 2014). Therefore, the findings of this thesis did not only reveal a yet unknown molecular cascade related to memory, but its alteration may underlie specific neurodegenerative disorders.

5 References

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Annex

Content

6 Isolation of neuronal nuclei from mouse hippocampal tissue for cell-type specific DNA (hydroxy)methylation analysis

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6.1 Abstract

The brain is a highly heterogeneous organ composed of various neuronal and glial cells making it difficult to characterize cell-type specific epigenetic marks. Each brain cell-types are known to have distinct epigenetic signatures, which mediate cell-type specific regulation of gene expression. To overcome this problem, we optimized a method for the isolation of neuronal and non neuronal cells from murine brain tissue using a fluorescence-labeled anti-NeuN antibody followed by flow cytometry. Isolated nuclei can be used for the analysis of epigenetic marks that decorate the DNA, such as 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC).

6.2 Introduction

The goal of the protocol described below can be used to isolate individual cell types from brain tissue for subsequent analysis of epigenetic marks present at the DNA, including 5mC and 5hmC. This protocol relies on a specific antibody that selectively recognized mammalian neuronal nuclei (NeuN) named anti-NeuN. Anti-NeuN antibody targets FOX3, a splicing factor present at nuclear speckles in neurons (Dent et al. 2010). Flurorescence-activated cell sorting

(FACS) is a novel technique in neuroscience to distinguish between neuronal from non-neuronal cells. To date, very few research groups have incorporated FACS in their studies for the analysis of epigenetic modifications, mainly because it is difficult to dissociate neural cells without causing cellular damage. In particular, adult brain tissues contain large amounts of connective tissue, which yield few viable cells. Here, we successfully developed a quick and efficient protocol for the isolation of neuronal nuclei from brain tissues that does not require ultracentrifugation.

6.3 Methods

Animals. C57Bl/6J mice were maintained under a reverse light-dark cycle in a temperature and humidity-controlled facility with food and water *ad libitum*. All experimental manipulations were performed during the animals' active cycle in accordance with guidelines and regulations of the cantonal veterinary office, Zurich. All behavioral tests were conducted in adult male animals by experimenters blind to treatment.

Tissue collection. Immediately after sacrifice, the brain was removed and the hippocampus rapidly dissected on ice-cold Hank's balanced salt solution (HBSS) without calcium and magnesium ions. Each hippocampus was minced with razor blades in approximately 1 mm thick sections and placed in a 15 mL conical centrifuge tube containing 1mL of cold HBSS.

Tissue dissociation and immunolabeling of dissociated cells. HBSS was replaced with 0.5 mL Accutase (Millipore, L11-007) and directly incubated at 37°C for 10 min in a waterbath. To stop the enzymatic digestion, 4 mL of Dulbecco's modified eagle medium (DMEM) was added and tissues were dissociated with a fire-polished Pasteur pipette. Triturated tissues were centrifuged for 5 min at 500 rcf and the pellet was carefully resuspended in 1mL HBSS. Cells were fixed with 4 mL of cold absolute ethanol (added drop-wise) under gentle vortexation and kept at -20°C for 10 min. Cells were then

pelleted by centrifugation at 500 rcf for 5 min and re-hydrated with 5 mL HBSS for 15 min at room temperature. Fixed cells were pelleted again by centrifugation at 500 rcf for 5 min and finally resuspended in 0.5 mL HBSS. At this point, cells were transfered to a 2 mL eppendorf tube and incubated with anti-NeuN antibody conjugated to Alexa Fluor® 488 (Millipore, MAB377) diluted 1 :2000 in 0.1% NP-40, HBSS for 30 min at 4°C (end-over-end rotation). For the optimization of this protocol, cells were stained with 2 μl of propidium iodide (1 mg/mL in 0.1% NP-40) to evaluate cell viability for 15 min at room temperature. Then, cells were transfered in a 15 mL conical centrifuge tube and centrifuged at 500 rcf for 5 min. Following supernatant removal, the cell suspension was resusprended in 1mL HBSS, filtered through a cell strainer (previously soaked with HBSS) under centrifugation (100 rcf for 5 seconds). All centrifugation steps until FACS were performed at 4°C.

Flow cytometry. A FACS Aria (BD Biosciences) instrument was used for cell sorting, and FACS CantoII (BD Biosciences) instrument was used for analysis without sorting (flow cytometry facility of the University of Zurich). Sorted cells were always kept at 4°C and collected into low-binding microfuge tubes. NeuN-positive and NeuN-negative sorted cells were centrifuged at 300×g for 5 min.

DNA extraction. DNA was extracted from pellets using the AllPrep DNA/RNA Mini kit (Qiagen) according to manufacturer's instructions. DNA concentration was determined using the Qubit® dsDNA High Sensitivity assay kit and Qubit® Fluorometer.

6.4 Results

Dissociation of hippocampi.

The dissociation of hippocampi was performed in an accutase solution, which contains a mixture of proteolytic and collagenolytic enzymes. The incubation period was optimized and set to 10 min, as this produced a high number of viable cells with minimal damages. Cells were subsequently triturated through Pasteur pipettes with tip fire-polished to an opening of 1 and 0.5 mm.

Fixation and immunolabeling.

As the anti-NeuN antibody recognizes an intracellular neuronal marker, both fixation and permeabilization of cells are crucial. To do so, we used 80% ice-cold ethanol added in a drop wise manner while mixing gently on a vortex. This ensures fixation and minimizes the formation of cell clumps. Cells are fixed for 10 min at -20 °C. Fixed cells were incubated with an anti-NeuN antibody conjugated to Alexa Fluor® 488 at a dilution of 1 : 2000. Antibody dilution was optimized so that two distinct populations were distinguished : FITC-positive and FITC-negative.

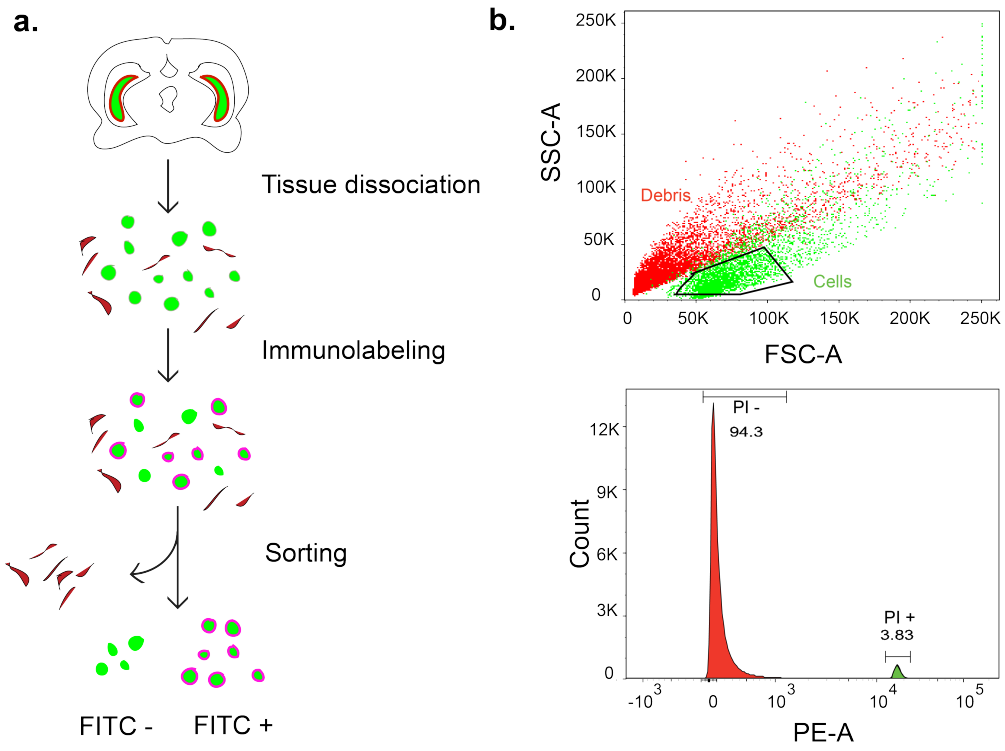

Figure 6-1 a. Schematic protocol for the isolation of neuronal nuclei. **b**. Scatter plot showing distinct clusters for debris and cells (upper panel). Each dot represents one event detected by the laser. Forward scatter (FSC) represents size of the event; side scatter (SSC) represents granularity of the event. The box around cells indicates events that were "gated" for subsequent fluorescence analysis. Histogram represents the fluorescence signal for propidium iodide (PI) from fixed adult mouse hippocampus (lower panel).

FACS analysis

During FACS, single cells pass through the path of a laser; light passing through the sample (forward scatter: FSC) represents size of the event, and light reflected at a 90° angle (side scatter: SSC) represents granularity of the event. In a light scatter plot (Fig. 6-1), each dot represents one event (either a cell or piece of debris). Events of a similar type tend to cluster due to their unique property of FSC and SSC. To distinguish between cell bodies and debris, we took advantage of propidium iodide (PI), a fluorescent intercalating agent that can be used to stain DNA. After membrane permeabilization, PI

can easily penetrate cell membranes. Therefore, by measuring the emmitted flurorescence of PI, we can differentiate cell bodies from debris (Fig. 6-1). The gate was set in a FCS area/ SSC area dot plot that contains events corresponding to cell bodies (P1). The fraction containing cell bodies may contain cell doublets or clumps, which can affect the purity of the sorting. To select single cells only, a second gating step (P2) was performed in the FSC height versus FSC area. Events distributed along the diagonal are the single cells. To separate NeuN-positive from NeuN-negative cells, these events were further plotted according to their Alexa Fluro 488 (FITC)-area versus FSC-area properties (Fig. 6-2).

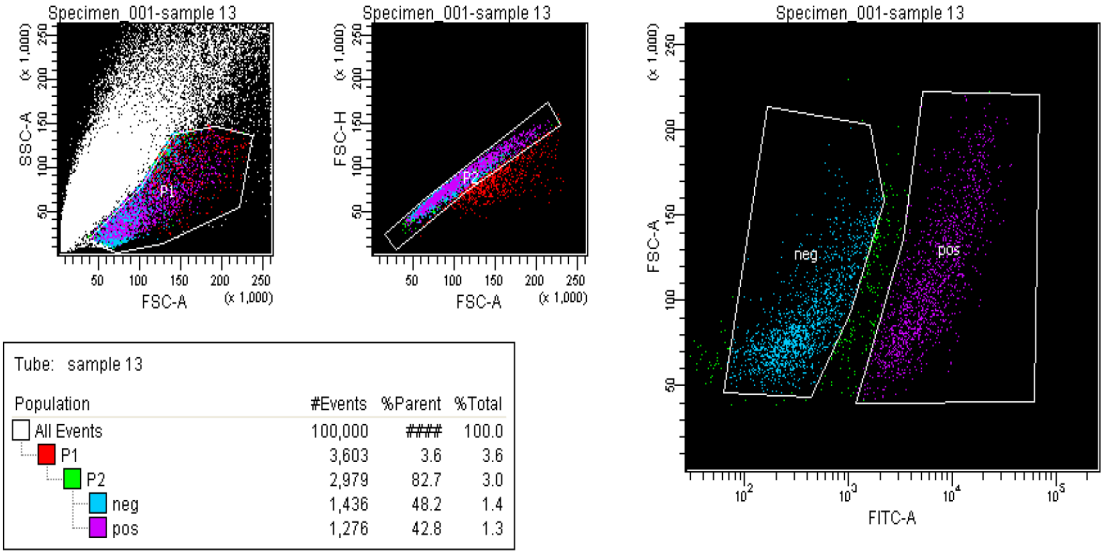

Figure 6-2 Flow cytometry analysis of neuronal nuclei dissociated from the mouse hippocampus using the neuron-specific antibody, NeuN conjugated to Alexa fluoro- 488 (FITC). Cells were first sorted based on their forward and side scatter from all possible events (left panel). This gate is called P1 (population 1). Next, single cells were sorted based on their size from the doublets or larger clumps of cells. This gate is called P2 (middle panel). Single cells were further gated as either non-neuronal cells (FITC neg) or neuronal cells (FITC pos) (right panel).

Two cell populations were distinguished : NeuN-positive and NeuN-negative cells, and each population were subsequently collected. We further examined sorted NeuN-positive and NeuN-negative cells with light microscopy and

observed round cell bodies with few debris (data not shown). Typically, we obtained an average of 300 000 NeuN-positive cells and 260 000 NeuN-negative cells per two hippocampal hemispheres.

6.5 Discussion

This FACS procedure has many potential applications in neuroscience. It could be valuable for separation of neuronal nuclei from other brain regions and may be applicable to other mammalian species. One major limitation concerns the relatively low yield of isolated cells, especially if the brain region analyzed is small in size. In this case, pooling of brain regions from a number of animals may be necessary. The use of the anti-NeuN antibody requires the fixation and permeabilization of tissue so that it can efficiently access the nucleus. During the fixation and permeabilization procedures, cell membranes are damaged, thus cytoplasmic RNAs and proteins can leak from the cell. This affects subsequent RNA extraction as only RNAs that remain in the nucleus will be quantifiable. Consistently, this protocol does not allow the isolation of proteins. Nevertheless, this protocol provides a simple and effective way to isolate neuronal from non-neuronal cells for subsequent analysis of DNA epigenetic modifications. It can be easily used in combination

6.6 Contributions

E.A.K and L.v Z jointly developed and optimized this protocol.

6.7 References

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7 The microRNA cluster miR-183/96/182 contributes to long-term memory in a protein phosphatase 1-dependent manner

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7.1 Abstract

Memory formation is a complex cognitive function regulated by coordinated synaptic and nuclear processes in neurons. In mammals, it is controlled by multiple molecular activators and suppressors, including the key signalling regulator, protein phosphatase 1 (PP1). Here, we show that memory control by PP1 involves the miR-183/96/182 cluster and its selective regulation during memory formation. Inhibiting nuclear PP1 in the mouse brain, or training on an object recognition task similarly increases miR-183/96/182 expression in the hippocampus. Mimicking this increase by miR-183/96/182 overexpression

enhances object memory, while knocking-down endogenous miR-183/96/182 impairs it. This effect involves the modulation of several plasticity-related genes, with HDAC9 identified as an important functional target. Further, PP1 controls miR-183/96/182 in a transcription-independent manner through the processing of their precursors. These findings provide novel evidence for a role of miRNAs in memory formation and suggest the implication of PP1 in miRNAs processing in the adult brain.

7.2 Contribution

E.A.K designed and conducted experiments together with A.J which led to Figures 3 and 6b.

8 Impaired PP1-dependent biogenesis of miR-183/96/182 underlies cognitive dysfunctions associated with aging and TDP-43 pathologies

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8.1 Abstract

Cognitive decline in aging is a normal process that can become pathological and lead to dementia. Here, we show in mice that aged-related memory decline is caused by a defect in the biogenesis of the microRNA cluster miR-183/96/182 resulting from an increase in protein phosphatase 1 (PP1), a potent memory suppressor. Correction of the defect restores normal memory in aged animals. Similar alterations in miR-183/96/182 and PP1 affect patients with TDP-43 pathologies. These results suggest a novel mechanism involving non-coding RNAs in cognitive disorders.

8.2 Contribution

E.A.K conducted the environmental enrichment experiment used in Figures 1f and Supplementary Figure 8. E.A.K further designed/performed molecular cloning presented in Figures 2a, and Supplementary Figures 4 and 7.

9 Subregion-Specific Proteomic Signature in the Hippocampus for Memory Formation in Adult Mice

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9.1 Abstract

The hippocampal formation is a brain structure essential for higher-order cognitive functions. It has exquisite differences in anatomical organization and cellular composition, and hippocampal sub-regions have different properties and functional roles. Areas CA1 and CA3 in particular, are key sub-regions for learning and memory formation that fulfill complementary but specific functions. The molecular basis for such specific properties and the link to learning and memory remain unknown. Here using a SWATH-MS proteomic approach and bioinformatic tools, we identify a selective proteomic signature in area CA1 and CA3, and reveal their specific dynamics during memory formation. We show that 30% of all quantifiable proteins are differentially expressed in area CA1 and CA3 at baseline, and that each proteome responds differently during the formation of memory for object or object location. Using clustering and cross-correlational analyses, we outline specific

temporal proteomic profiles and an increased correlation between both forms of memory within area CA1, but not within area CA3. These results provide new insight into a proteomic basis for hippocampal sub-region molecular and functional specificity.

9.2 Contribution

E.A.K performed behavioral experiments (object recognition) used in this study.

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In particular, I need to highlight my collaboration and friendship with Ali. Together, we published two scientific articles on PP1 and wrote a book chapter. Beyond our scientific achievements, we shared wonderful moments in and outside the lab that won't be forgotten. Additional thanks goes to my colleague and friend Lukas. Together, we spent a great deal of time developing a protocol for sorting hippocampal neurons from tissue. I am further grateful to him for seeing lab work through such a pragmatic lens.

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But also the wonderful human beings in my private sphere *i.e.* Clara, Jacques, Christelle, Sabine, Jenn, Delphine, Elodie, Chloé and Jérémy, Fabian, Jens, Heidi and Richard.

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